UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS
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TESIS DOCTORAL

Hipótesis sobre el origen y la función de la secreción de mucílago en semillas de especies Mediterráneas
Mucilage secretion in seeds of Mediterranean species : hypotheses about its origin and function

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

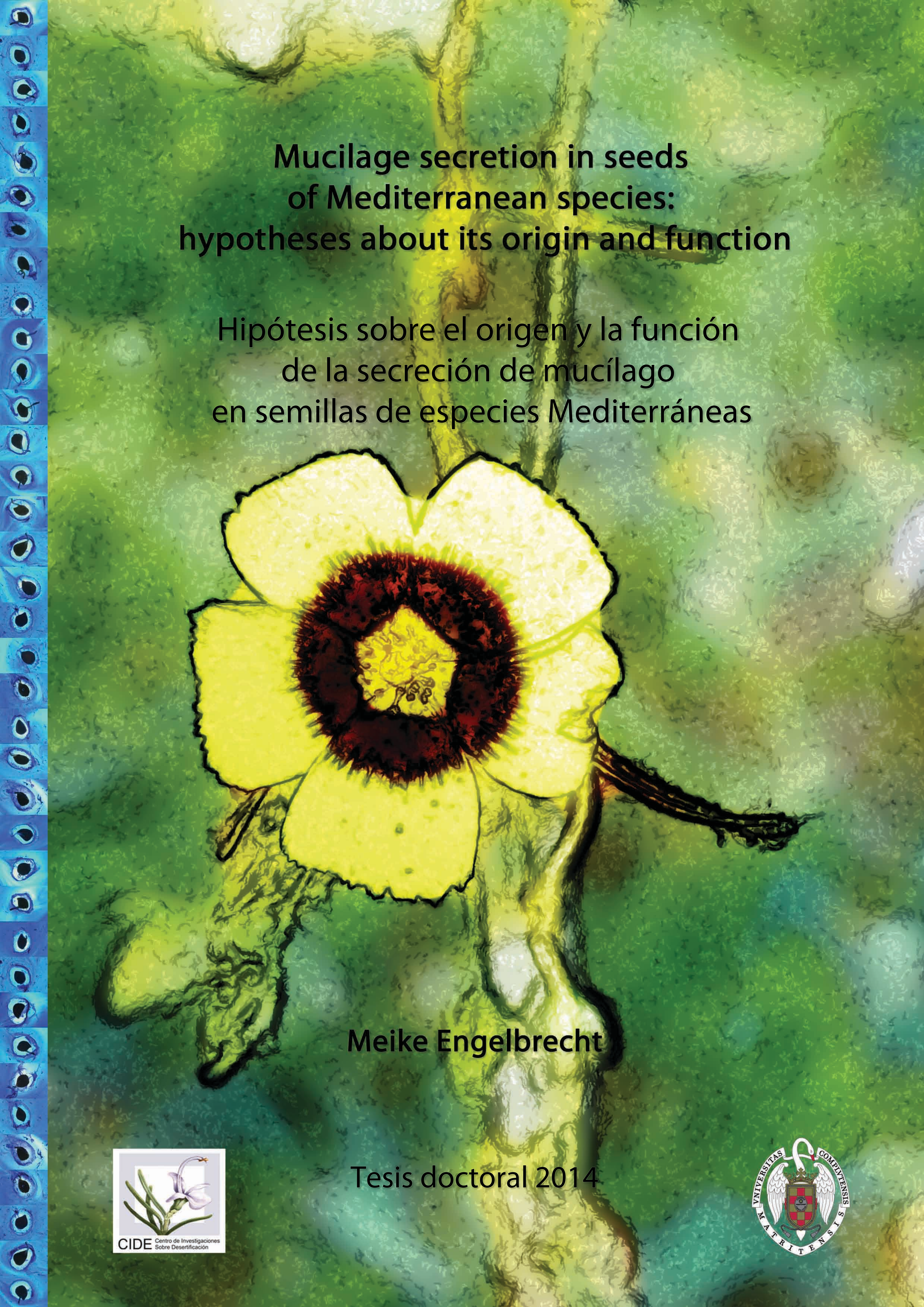
PRESENTADA POR

Meike Engelbrecht

Director

Patricio García-Fayos Poveda

Madrid, 2014



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Meike Engelbrecht

Tesis doctoral 2014

CIDE Centro de Investigaciones Sobre Desertificación

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HIPÓTESIS SOBRE EL ORIGEN Y LA FUNCIÓN DE LA SECRECIÓN DE MUCÍLAGO EN SEMILLAS DE ESPECIES MEDITERRÁNEAS

DISSERTATION

THESIS DOCTORAL

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Sobre Desertificación

UNIVERSIDAD COMPLUTENSE
MADRID

Dr. Patricio García-Fayos Poveda, Investigador Científico del Centro de Investigaciones sobre Desertificación (CIDE) del Consejo Superior de Investigaciones Científicas (CSIC) certifica

Que la memoria adjunta titulada "Hipótesis sobre el origen y la función de la secreción de mucílago en semillas de especies Mediterráneas - Mucilage secretion in seeds of Mediterranean species: hypotheses about its origin and function" presentada por Meike Engelbrecht ha sido realizada bajo mi inmediata dirección y cumple las condiciones exigidas para optar al grado de Doctor en Biología por la Universidad Complutense de Madrid.

Y para autorizar su presentación y evaluación por el tribunal correspondiente, firman el presente certificado a día 10 de Marzo de 2014

Dr. Patricio García-Fayos Poveda   Meike Engelbrecht

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ABSTRACT

An outstanding modification of the diaspore surface structure in many species of the Angiosperms is the presence of a mucilaginous layer. In these species, when the dry seed coat or the pericarp comes in contact with water, imbibes the outer cell wall completely and release a mucilaginous substance; a phenomenon known as myxospermy. Mucilage is composed of polysaccharides, mostly of pectins, and forms a gel like envelop around the diaspore that holds a considerable amount of water due to its hydrophilic nature. The seed weight as well as the volume increases significantly once the mucilage is released. Once it dries up, mucilage becomes stiff then gluing the diaspore to the surface on which it settles.

There are differences in mucilage composition depending on the species. However, the main component of the mucilage of the pericarp and seed coat in all species are pectins. The polysaccharide and acidic qualities of mucilage make them very hydrophilic so in the presence of water they hydrate rapidly, thus forming super absorbent hydrogels. After water absorption, the mucilage breaks through the cell wall forming the mucilaginous envelope surrounding the seed. In addition to pectin, mucilage in some species also contains strands of elementary fibrils of cellulose of different widths embedded in the pectin envelope. Mucilage can therefore be distinguished in “true” mucilage consisting almost exclusively of pectin, and “cellulosic” mucilage, which, additionally to pectin, also contains cellulose fibrils. Cellulosic mucilage seems to add an additional strength to the pectin mucilage layer and has been hypothesized that it prevents mucilage of being washed away from the seed coat or fruit pericarp making the mucilage more rigid and thus, resulting in an enhanced adhesion of the mucilage to the diaspore.

Mucilage release after wetting in seeds and fruits is a common feature in many families of Angiosperms. From bibliographical references we found that 1369 species of 102 plant families, belonging to 40 different orders have diaspores which secrete mucilage on wetting.

Recently it has been proposed that seed mucilage could be an evolutionary advanced trait because a relation between the ordinal phylogenetic position of plant families and the frequency of myxodiasporic taxa they comprise was found. However, this relation only occurs if the absolute numbers of myxodiasporic taxa are taken into account. There are families with a very large amount of species in contrast to families with a low number of species, thus it is much more likely to find references for myxodiaspory in those families. Also, in many cases, the larger families are usually those in which more research has been conducted resulting in a higher rate of mucilage discovery.

Several different functions have been proposed for myxodiaspory which can be grouped under two main hypotheses. One related to seed germination and the other one related to seed dispersal. As mucilage absorbs rapidly a big amount of water and retains it during a certain time period, it has been hypothesized that mucilage should serve as a way to absorb and store water for germination. Contrarily, several authors also found that the presence of mucilage inhibited the germination in some other species. The ability to absorb and store water has furthermore been discussed to enhance seed survival under strong climatic conditions as mucilage secretion can be activated by morning dew. The absorbed moisture might not trigger germination but can promote other processes in the embryo, such as repair and restoration of the DNA of the embryo damaged by insolation.

The functions of myxodiaspory have also been frequently discussed in relation to seed dispersal as the hydrated mucilage coat is extremely sticky, and, once it dries up, works effectively as glue. Two main roles have been recognized for it, first, regarding to the adhesion of the diaspore to the fur and feathers of animals, functioning as a dispersal mechanism (telechory), and second, regarding to its power of gluing the seed to the ground, thus working as anti-dispersal mechanism (antitelechory). In this sense myxodiaspory has been proven to reduce seed removal in semiarid and arid environments. Seeds deposited after dispersal on the soil surface of steeped slopes are then at a risk of being removed downslope with runoff water towards the lower parts of the slopes or in the valley bottoms where they can get buried or be affected by a stronger competition of seedlings than on the upper or medium parts of the slopes. On slopes the removal of diaspores by soil erosion can be a major difficulty to overcome in low competitive plants and, in consequence, anchoring the diaspore to the ground near the mother plant may be a favourable adaptation to avoid strong competition. However, in this scenario neither the role of this mechanism in the assembly of plant communities of eroded areas nor its adaptive character has been studied.

Gluing the seed to the ground therefore not only hinders further movement by soil erosion, but additionally prevents massive seed collection by animals. Seed collection by granivorous animals was significantly reduced when seeds were glued to the soil or its seed mass incremented due to adhered soil particle on the mucilage coat, which at the same time provoked a camouflaging effect for the seed.

Mucilage secretion can probably not be reduced to only one single function and conflicting ideas about its ecological role may depend on the plant species studied. However, in some plant groups the influence of mucilage on the germination ability of seeds could be excluded if other seed characteristics, such as long dormancy, also apply. There are many species with physical dormancy in which the primary reason for the delay of germination is the lack of permeability of the seed (or fruit) coat to water. In the species of these families, seed mucilage seems therefore to be not very relevant for germination and it would be more likely that mucilage secretion played a function related to seed dispersal.

In the present memory we present and discuss the results obtained in the study about origin and function of seed mucilage in the Cistaceae, a family of Mediterranean plants that live in open and degraded shrublands. Briefly, they are:

In the first chapter, it was tested whether mucilage secretion can be considered a selective response to soil erosion in plant species inhabiting semiarid environments. The amount and type of mucilage secretion by seeds of *Helianthemum violaceum* and *Fumana ericifolia* (Cistaceae) was related with the number of raindrop impacts needed to remove these seeds after gluing them with their own mucilage to the ground as well as the time that these seeds resist water runoff without detaching. Also the amount of seed mucilage production by plants growing in habitats without erosion and plants affected by severe erosion by fitting mixed effect models was compared. The results show an important phenotypic variation in the amount of mucilage secretion in both species; but suggest that the effect of mucilage secretion in the rate of seed removal by erosion is species and mechanism dependent. For *F. ericifolia*, the amount of mucilage secreted by the seeds is directly proportional to their resistance to raindrop impacts and is positively related to the intensity of the erosive processes that the plants experience. Nevertheless, all the seeds resist the force of runoff during 60 minutes irrespective of the amount of mucilage they produce. In *H. violaceum*, mucilage secretion *per se*, and not the amount of mucilage produced by the seeds has an effect on the rate of seed removal by erosive processes. Furthermore, cellulosic fibrils were found only in the mucilage of *F. ericifolia* but not in *H. violaceum*. Overall, these results only partially support the hypothesis that a selective response to soil erosion exist.

In the second chapter, a test of Ellner-Shmida's hypothesis was performed, that in semiarid environments aridity may select for the lack of seed dispersal mechanisms (atelechory) in many plants, whereas post-dispersal selective forces such as soil erosion, seed predation or limitations to water uptake by seeds may select for structures facilitating seed anchorage to the ground (antitelechory). Therefore the proportions of species with seed anchorage mechanisms and that of atelechoric species in shrubland colonizing flat areas and hillslopes in two sites differing in climate dryness were analyzed. Their relation with several soil properties involved in runoff generation, seed-soil contact and water uptake by seeds and with nest density of granivorous ants was also explored. The results support the hypothesis that in semiarid shrubland the proportion of species with seed anchorage mechanisms increases because of soil erosion but not because of climate dryness. This is the first time that a direct relation between the proportion of species with seed anchorage mechanisms and soil erosion is shown in plant communities; supporting the view that soil erosion shapes species composition in communities.

In the third chapter, the importance of mucilage secretion in seeds was evaluated as a mechanism to reduce seed collection by ants. Post-dispersal seed predation is a risk for plants in semiarid environments, leading to strategies to protect their propagules from seed collection by animals. Therefore three Mediterranean species with strong mucilage secretion on their seeds which become sticky upon wetting were selected. Seeds of *Rosmarinus officinalis*, *Fumana ericoides* and *Fumana thymifolia* were exposed to ants and survival was compared between dry loose seeds and seeds glued to the soil with previously secreted mucilage. The ant-plant interaction was analysed by scrutinizing seed collection by ants and by analysing the waste piles of ant nests. To test survival, groups of 10 seeds were placed on the ground. Each group consisted of 5 control (dry) and 5 mucilaginous seeds (previously mucilage secreted) and was covered by the cover of a Petri dish modified to permit only the entry of ants. Seeds were inspected weekly for seed disappearance and the survival function (Kaplan-Meier estimator –KM) was calculated. Seeds of the target species were important food items for ants and were actively collected, and more than 50% of the experimental seeds that were glued to the ground with their own mucilage survived at the end of the study period but only 0-20% of the control seeds survived after the same time of exposure. This seems to have positive implications for plant establishment for the studied species.

In the fourth chapter, the phylogenetic relationship of 19 *Fumana* species was constructed based on three molecular markers with coding (*matK*) and spacer (*trnT-L*) sequences of plastid and nucleic (ITS) DNA. The genus *Fumana* is one of the most diverse and least studied genera of the Cistaceae family with 21 recognized species with high morphological diversity, divided into three subgenera (*Fumana*, *Fumanopsis* and *Pomelina*). Bayesian Inference, Maximum Parsimony and Maximum Likelihood analysis as well as an estimation of the divergence times (BEAST) were applied. Phylogenetic relationships based on the plastid markers confirmed the monophyly of the genus. Results do not support the traditionally established infrageneric divisions, but confirm the presence of two main groups of species. Each clade clusters species differing in vegetative and reproductive characters and having been formerly grouped in three subgenera (*Fumana*, *Fumanopsis* and *Pomelina*). However, none of the clades clustered species exclusively from one of the recognized subgenera. Given the impossibility of defining morphologic characters which are common to all species of every clade we reject all infragenetic divisions and discard the existence of three subgenera. Significant ancestral character states were found in five cases (leaf form and leaf margin, glandular trichomes, diaspore and seed mucilage secretion), suggesting an adaptation to the Mediterranean environment and climate. A strong mucilage secretion was detected to be the most likely ancestral character state changing to a weak and absent state in four more recently separated species. Furthermore divergence times of *Fumana* date to around 16.97 Ma ago (24-10 Ma), with the divergence of major clades between the Middle and Late Miocene (15.61-8.8 Ma).

In the fifth chapter, a phylogenetic approach to study mucilage seed evolution was applied and the hypothesis that the presence of seed mucilage in the Cistaceae is an ancestral character state related to the ability of species to colonize eroded slopes tested. The family Cistaceae provides a good opportunity to study the evolution of this character because there is a large variability of mucilage occurrence within this family and a well solved phylogeny available. To achieve these objectives the presence of the character seed mucilage was mapped along the phylogeny of the Cistaceae, and the relation of this character with traits associated to the competitive ability of the plants, such as relative growth rate (RGR), seed longevity and seed size of seven representative species of the family analysed. These results were discussed in the light of the environmental changes that occurred along the history of the family. The results show that seed mucilage secretion in the Cistaceae is the most likely ancestral character state and when the type of mucilage (cellulosic and pectin) was included in the analysis, pectin mucilage seems to be the ancestral character state in the family. Seed mucilage was lost several times throughout the family but the most noticeable change is the total loss in the genus of *Cistus*. A pattern was found in the Cistaceae from low competitive species with strong mucilage secretion of cellulosic type, low RGR values, short seed longevities and big seed sizes that characterize the oldest genus (i.e. *Fumana*) towards the most modern species with a higher competitive ability, no mucilage secretion, higher RGR and seeds with longer viability and smaller sizes in the species of *Cistus*.

The present study on mucilage secretion by seeds showed clearly that myxodiaspory functions as antitelechoric mechanism in several species of the Cistaceae, as the mucilage effectively glued the seeds to the ground thus hindering further movement under simulated erosive conditions (drop impact and runoff) and preventing seed collection by granivorous ants. Furthermore, when testing the hypothesis of Ellner and Shmida, a higher frequency of antitelechoric species in plant communities of areas affected by erosive pressures in comparison to those communities of flat areas without soil erosion was found, but the frequency of this dispersal mechanism was not influenced by the increase of aridity. These results supporting the hypothesis that myxodiaspory is not an adaptation to aridity per se; instead, it should be considered a side effect to the consequence of this aridity. Regarding the evolutionary history of mucilage in the family of the Cistaceae, we found that the most ancestral character state is the presence of mucilage while the loss of mucilage is a derived character. The same pattern was found when focusing on the genus *Fumana*, in which a strong mucilage secretion was revealed to be the most ancestral state with a shift towards weak and absent mucilage in some of the more recent species.

RESUMEN

Introducción

Formación y composición de mucílago

En las semillas y frutos maduros, la cubierta de la semilla o del pericarpio, respectivamente, funciona como una protección del embrión contra la desecación temprana y el daño físico o biológico. La testa de la semilla o el pericarpio del fruto también pueden actuar como regulador de la absorción de agua, o del inicio de la germinación en algunas especies, o del mantenimiento de la latencia de las semillas mediante la prevención de la absorción de agua y el intercambio de gases en otras. En unos pocos casos, se ha comprobado que la cubierta de la semilla funciona como tejido de reserva durante la germinación. Además, en muchas especies la cubierta de la semilla o el pericarpio frecuentemente desempeña un papel en la dispersión de semillas mediante la modificación de sus propiedades o el desarrollo de estructuras especializadas.

Una modificación destacada en la estructura de la superficie de la diáspora en muchas especies de angiospermas es la presencia de una capa mucilaginosa. En estas especies, cuando la testa de la semilla o el pericarpio del fruto entran en contacto con el agua, la capa celular externa se humedece por completo y libera una sustancia mucilaginosa; un fenómeno conocido como mixospermia. El mucílago se compone principalmente de polisacáridos y forma una cubierta gelatinosa alrededor de la diáspora, que absorbe y mantiene una cantidad considerable de agua debido a su naturaleza hidrófila. Con la secreción de mucílago, tanto el peso de la semilla como el volumen de la misma aumentan significativamente. Cuando se seca, el mucílago se vuelve rígido y pega la diáspora a la superficie sobre la que se asienta.

Dependiendo de la especie hay diferencias en la composición del mucílago. Sin embargo, en todas las especies el componente principal del mucílago del pericarpio y de la cubierta de la semilla es la pectina. La mayoría de estudios sobre la composición del mucílago en semillas se han enfocado en la *Arabidopsis thaliana*, pero también se ha investigado su composición en las semillas de albahaca (*Ocimum* ssp.), de lino (*Linum* ssp.) y de plantago (*Plantago* ssp.)

La pectina está constituida por un grupo heterogéneo de polisacáridos ácidos que se caracteriza por la presencia de ácido galacturónico (GalA). Para simplificar, la estructura principal de la pectina comprende dos tipos de polímeros: ramnogalacturonano I (RG I) y homogalacturonan (HG). Analizando los azúcares presentes en el mucílago, se encontraron cantidades significativas de azúcares neutros como ramnosa (Rha), pero también se detectaron principalmente galactosa (Gal) y glucosa (Glc) así como fucosa (Fuc), arabinosa (Ara), xilosa (Xyl) y manosa (Man) [34-48 % (w / w) de los azúcares neutros y ácidos totales en mucílago]. Los polisacáridos y su carácter ácido hacen que el mucílago sea muy hidrófilo, de modo que se hidratan muy rápido en presencia de agua, formando hidrogeles súper absorbentes. Eso permite que el mucílago rompa a través de las paredes de las células de la epidermis y facilite la absorción y retención de agua alrededor de la semilla.

En algunas especies el mucílago, además de pectina, contiene filamentos de fibrillas de celulosa de diferentes anchuras asociados e integrados en la pectina. Por tanto, el mucílago se puede catalogar como "verdadero” mucílago, que se compone en la mayoría de las especies casi exclusivamente de pectina y mucílago "celulósico" que, además de la pectina, contiene también micro fibrillas de celulosa. Parece que el mucílago celulósico añade resistencia adicional a la capa de mucílago pectínico, y se ha sugerido que evita que el mucílago se lave de la testa de la semilla o del pericarpio de la fruta, produciendo una mayor rigidez que resulta en una mejor adherencia del mucílago a la diáspora. Esta diferencia estructural también influye en la fuerza de adherencia de la diáspora a cualquier superficie, por lo tanto, ayuda a su transporte en la piel o plumas de los animales, previene la remoción de las semillas por la erosión y su depredación por animales y también ayuda a que la raíz penetre en el suelo.

El mucílago con fibrillas de celulosa se ha encontrado en varias familias de plantas, entre otras, en Asteraceae, Brassicaceae, Cistaceae (genus Fumana), Euphorbiaceae , Lamiaceae y Polemoniaceae mientras que el mucílago puro o pectínico se ha encontrado, entre otras, en la familia de las Linaceae, Plantaginaceae, Poaceae y Cistaceae (genus Helianthemum). Simples métodos de teñir como ha recopilado algunos autores ayudan a distinguir entre los dos tipos de mucílago. El rojo de rutenio y la safranina proporcionan reacciones muy fuertes de tinción en la pectina, en la que el rojo de rutenio típicamente revela su estructura homogénea. La tinción con azul de metileno o I en KI+H2SO4 hace visible la presencia de fibrillas de celulosa en la matriz de la pectina.

La producción de mucílago de semilla en la *Arabidopsis thaliana* es parte de un proceso de diferenciación excepcional durante el cual las células de la epidermis del óvulo maduro crecen, reorganizan su citoplasma, sintetizan y secretan el mucílago, y forman una pared celular secundaria. Después de una fase de producción extensiva de mucílago, éste se localiza entre la membrana plasmática y la pared periclinal exterior de la célula de la epidermis, lo que resulta en la compresión del protoplasto. Esta reducción del protoplasto da lugar a la formación de una columna citoplasmática en forma de volcán que se encuentra bajo el bolsillo de mucílago. La síntesis de una pared celular celulósica para rodear la columna citoplásmica resulta en la formación de la columella.

En la mayoría de las especies el mucílago se origina de las células especializadas de la epidermis de la semilla, pero también puede estar presente en la epidermis y las células sub-epidérmicas o únicamente en estas últimas. Por otra parte, en algunas especies de la familia de las Lythraceae, el mucílago solo está presente en ciertas partes de la semilla, en otros casos se encuentra en sacos dentro de la testa de la semilla, como en la familia de las Sterculiaceae o incluso la capa de mucílago puede ser discontinua porque las células que lo contienen se encuentran dispersas entre las células sin mucílago (Lamiaceae). También las células mucilaginosas pueden estar presentes en la punta de pelos de la diaspora, que pueden ser unicelulares como en la *Ruellia* o multicelulares como en la *Blepharis ciliaris* o en la *Blepharis persica*. Además, el mucílago también se puede encontrar en el pericarpio de la diaspora como ocurre en las Asteraceae, Lamiaceae, Piperaceae o Urticaceae. Cuando el mucílago es producido por el pericarpio, el mucílago se denomina mixocárpico mientras que hablamos de mixospérmico para referirnos a la producción de mucílago por las semillas.

Origen y linaje del mucílago

La producción de mucílago en las semillas y los frutos después de la humectación es una característica común en muchas familias de angiospermas. En las referencias bibliográficas encontramos 1.369 especies de 102 familias de plantas, pertenecientes a 40 órdenes diferentes que tienen diásporas que secretan mucílago una vez humectadas. La mayoría de estas especies se encontraron en las familias de las

Asteraceae (197 especies), Lamiaceae (158), Acanthaceae (144), Fabaceae (86), Brassicaceae (81), Cistaceae (76), Euphorbiaceae (52), Polemoniaceae (42), Plantaginaceae (32), Malvaceae (30), Podostemaceae (20), Linaceae (19), Rosaceae (18), Araceae (16), Rutaceae (16), Arecaceae (16), Scrophulariaceae (16), Poaceae (15), Solanaceae (15), Violaceae (14), Nyctaginaceae (13) y seguidas de otras 89 familias en las que encontramos, al menos, entre 1 y 10 especies con semillas o diásporas mucilaginosas.

Además de las referencias bibliográficas, en 2010 realicé en las instalaciones del Millennium Seed Bank - Royal Botanic Gardens, Kew, una prueba de secreción de mucílago por diásporas en 237 especies pertenecientes a 95 familias de plantas, y comprobé que el 8,4 % de estas especies (20 especies), que pertenece al 9,5 % (10) de las familias testadas secretan mucílago. Cuando se suman la información bibliográfica y mi propio estudio, encontramos que un total de 108 familias de angiospermas poseen diásporas que secretan mucílago. En otras de las familias de plantas que fueron analizadas por nosotros y en algunas citas bibliográficas se encontró que las semillas no segregaron mucílago.

En un investigación reciente, algunos autores proponen que el mucílago de semilla debe ser un rasgo evolutivo avanzado debido a que encontraron una relación entre la posición filogenética ordinal de familias y la frecuencia de los taxones con aparición de semillas con mucílago. Además, al inspeccionar el hábitat de las familias con especies mixodiaspóricas, encontraron una mayor frecuencia de taxones que viven en hábitats secos en las familias filogenéticamente avanzadas (por ejemplo, Asteraceae, Brassicaceae y Poaceae) que en familias de los grupos basales de angiospermas (por ejemplo Nymphaeales y Magnolids en hábitats húmedos). Sin embargo, en su análisis los autores sólo representan el número absoluto de taxones mixodiaspóricos, y no sus números relativos. Así, hay familias con una gran cantidad de especies y otras con un bajo número de especies, por lo que es mucho más probable encontrar referencias a la mixodiasporia en aquellas familias. Además, generalmente, las familias sobre las que más investigaciones se han realizado son aquellas más grandes, resultando en una mayor tasa de descubrimientos de mucílago. Sin embargo proponemos que, dado que existen muchas especies con mucílago en muchas familias y órdenes a lo largo del árbol filogenético de las angiospermas, y ello unido a la evidencia paleontológica de que el mucílago de semillas ya existía en el Eoceno Medio, que el carácter del mucílago de semillas se podría considerar ancestral. El carácter del mucílago podría haberse perdido en algunos casos mientras que se ha conservado en otros. Se podría comprobar esta hipótesis en diferentes niveles del árbol filogenético de las angiospermas, entre órdenes y familias, o comparando géneros y especies dentro de familias. Por otra parte, el carácter ancestral de la mixodiasporia en un determinado grupo de plantas puede ser testado al mismo tiempo que las diferencias entre los tipos de mucílago, distinguiendo entre mucílago de solo pectina y mucílago celulósico. A pesar de que el resultado de un análisis ancestral realizado a un nivel más pequeño podría no revelar el origen real de mucílago en el árbol filogenético de las Angiospermas, abriría muchas oportunidades para discutir la función actual o formular hipótesis sobre una función del mucílago en el pasado, en semillas de estas familias o géneros.

Función de mucílago

Se han propuesto dos hipótesis principales para delimitar varias funciones diferentes del mucílago en semillas. Una hipótesis está relacionada con la germinación de la semilla y la otra relacionada con la dispersión de semillas.

El mucílago absorbe rápidamente una gran cantidad de agua y la retiene durante un cierto período de tiempo, por ello se ha planteado la hipótesis de que el mucílago podría servir como una forma de absorber y acumular agua para la germinación. Esto ha sido estudiado por un autor, que asocia la gran capa mucilaginosa de las semillas de *Carrichtera annua* con la posible capacidad de "sentir" el agua y regular la germinación. Otros autores argumentan que el mucílago favorece la germinación porque amplía la superficie de contacto entre la semilla y el sustrato, aumentando por tanto la difusión del agua.

Además se plantea que esta área de contacto ampliada entre la diáspora y el suelo minimiza la pérdida de agua de la semilla. Una germinación acelerada gracias a la presencia de mucílago se ha descrito para *Anastatica hierochuntica* y además se comprobó que semillas mucilaginosas de *A. sphaerocephala* mostraron una menor sensibilidad al estrés por un aumento en el potencial osmótico (PEG) y la salinidad (NaCl) que semillas sin mucílago.

Por el contrario, varios autores también apuntaron que la presencia de mucílago inhibió la germinación en algunas especies. Se manifestó que el exceso de agua alrededor de las semillas de *Blepharis persica* y *Spinacia oleracea* inhibió la germinación. Esto se interpretó como una prueba de que el mucílago limita el acceso de oxígeno a la semilla, ya que en estas especies la germinación mejoraba significativamente con la eliminación de la capa de mucílago o mediante el aumento de la concentración de oxígeno en el agua circundante. Del mismo modo, el aumento de grosor en el mucílago de las semillas de *Lesquerella perforata* y *L. stonensis* (Brassicaceae) se pudo correlacionar con una reducción en la tasa de germinación. Algunos autores interpretaron este fenómeno como un mecanismo de prevención de la germinación de semillas en hábitats áridos, donde la primera lluvia no proporciona suficiente agua para un desarrollo exitoso y por lo tanto puede no ser la mejor oportunidad para el establecimiento de las plantas.

El mucílago de la semilla no sólo se secreta bajo condiciones muy húmedas, sino también bajo una lluvia débil o incluso bajo el rocío de la noche, y posteriormente puede ser rehidratado después de secado. Estas condiciones normalmente no desencadenan la germinación, pero pueden promover otros procesos en el embrión, como la reparación y restauración del ADN. Las semillas depositadas en la superficie del suelo en condiciones desérticas pueden someterse a una insolación fuerte. Por lo tanto, se ha discutido si el mucílago de semillas mejora su supervivencia en condiciones climáticas extremas ya que el agua retenida aumenta la reparación del ADN del embrión de la semilla. En este contexto, el ADN de las semillas de dos especies de *Artemisa* se dañó artificialmente mediante radiación y fue analizada la influencia del mucílago en la reparación del ADN deteriorado. Encontraron una restauración del ADN en las semillas con una capa de mucílago intacta pero no en las semillas donde el mucílago se había eliminado.

Una vez germinadas, las plántulas jóvenes son susceptibles de muchos peligros tales como la desecación, los patógenos o la competencia con la vegetación existente. Hay diversos autores que proponen que el mucílago de semillas influye positivamente en el establecimiento de las plántulas, afirmando que la presencia de mucílago aumenta considerablemente la resistencia de las plántulas de *Artemisia spaerocephala* y *A. monosperma* en ambientes desérticos. Se encontró que, en frutos de *Cavanillesia platanifolia* (Bombacaceae), una capa de mucílago abundante era necesaria para una exitosa expansión de los cotiledones y el posterior crecimiento de plántulas en condiciones de riego infrecuente. Además, el mucílago redujo el grado de marchitamiento de plántulas, aumentando así las probabilidades de supervivencia de las mismas en condiciones de sequía. También se ha propuesto que algunos compuestos orgánicos presentes en el mucílago de la semilla pueden ser utilizados por el embrión para su desarrollo, ya que las plántulas de *Artemisia monosp*erma crecidas de semillas con una capa de mucílago intacta tenían mayor vigor que las que carecían de ella. Unos autores muestra que los polisacáridos del mucílago (o sus derivados) son absorbidos por las raíces y posteriormente transportados a los brotes de las plántulas, lo cual proporciona una evidencia directa de la absorción de moléculas derivadas del mucílago en el crecimiento temprano de las plántulas.

Las funciones de la mixodiasporia también se han discutido frecuentemente en relación a la dispersión de las semillas, dado que la capa de mucílago hidratado es extremadamente pegajosa y, una vez seca, funciona efectivamente como pegamento. Dos papeles principales han sido atribuidos a esta característica, en primer lugar, con respecto a la adhesión de la diáspora a la piel y plumas de los animales, lo que crea un mecanismo de dispersión (telecoria), y segundo, con respecto al poder de pegado de la semilla al suelo, funcionando así contra la dispersión (antitelecoria).

El primer papel reclamado por el carácter pegajoso de mucílago es el relacionado con la capacidad de dispersar la semilla a larga distancia. Se ha descrito que especies de *Euphorbia* en Hawái han sido dispersadas entre islas, pegadas en las alas de los pájaros gracias a la secreción de mucílago de las semillas. Por otra parte, las semillas de *Anastatica hierochuntica* son comidas por aves (*Phasianidae*) en el desierto de Negev, y al mismo tiempo es probable que se adhieran al cuerpo del ave y así se dispersen a lo largo de grandes distancias. Según un autor la dispersión a larga distancia de cierta Polemoniaceae (*Glieae* y *Polemoniae*) puede interpretarse como el resultado de ser transportada, pegada a los animales migratorios. Cuando las frutas y semillas mucilaginosas son consumidas por animales, no sólo pueden adherirse accidentalmente a estos y dispersase, sino que también se ha sugerido que el mucílago que cubre la semilla o fruto proporciona lubricación para el paso a través del tracto digestivo de los animales. La aptitud para la germinación de semillas comidas está inversamente relacionada con el tiempo que pasan en el tracto digestivo de los animales, y a continuación la excreción temprana evita daños a las semillas al mismo tiempo que asegura la dispersión a larga distancia. Esto ha sido descrito para las semillas de *Cecropia*, cuyos frutos componen una parte principal en la dieta de los murciélagos y cuyas células secretoras del mucílago se encuentran frecuentemente intactas en las heces de estos mamíferos. Se han observado otros animales que se alimentan de semillas con mucílago como la perdiz griega (*Alectoris gracea*), que consume la semilla de *Lepidium perfoliatum*, *Sisymbrium altissimum* y *Descurainia pinnata*.

El mucílago en las semillas funciona eficientemente como mecanismo antitelecórico. Diversas formas de mecanismos antitelecóricos están descritas para las plantas que viven en hábitats áridos, pero encontraron sólo dos mecanismos en los que la semilla misma restringe su dispersión secundaria en el espacio debido a que se adhiere a la tierra (mixospermia y trypanospermia). Diásporas con un mecanismo trypanospérmico tienen la capacidad de introducirse en el suelo debido a los mecanismos de perforación y giro producidos por la arista, vilano u otras estructuras de la semilla, mientras que diásporas mixospérmicas se pegan al suelo.

Los mecanismos antitelecóricos han sido normalmente relacionados con las condiciones climáticas donde habitan las plantas y han sido reportadas más frecuentemente en ambientes áridos y semiáridos que en ambientes húmedos. Se ha establecido la teoría del “mother-site” (sitio de la planta madre), argumentando que en hábitats áridos y semiáridos las proximidades de la planta madre generalmente tienen condiciones favorables y adecuadas para el crecimiento de las plántulas. Se ha argumentado que la dispersión a larga distancia para las especies que viven en esos ambientes ofrece muy pocas ventajas, ya que existen condiciones favorables cerca de la planta madre y se encuentran variaciones significativas en el clima y el medio biótico generalmente más lejos que la distancia de dispersión que la semilla puede lograr. En consecuencia, se ha demostrado que la secreción del mucílago que adhiere la semilla al suelo favorece el establecimiento del *Helianthemum squamatum*, por eso la proximidad a una fuente de semillas es el principal predictor para la emergencia de las plántulas.

Los ambientes áridos y semiáridos presentan diversas dificultades para el establecimiento de las plantas. En el Mediterráneo y ambientes semiáridos se produce con frecuencia la erosión del suelo en cuestas empinadas y las lluvias escasas se concentran a menudo en intensos eventos. Las semillas depositadas en la superficie del suelo de las laderas están en riesgo de ser arrastradas por la escorrentía del agua hacia las partes más bajas o hacia el fondo de los valles en los que pueden resultar enterradas o ser afectados por una competencia más fuerte que en las partes altas o medianas de las cuestas. Por lo tanto, la erosión en las laderas semiáridas puede suponer una dificultad importante a superar por las plantas poco competitivas y, en consecuencia, el anclaje de la diáspora al suelo cerca de la planta madre puede ser una adaptación favorable para evitar la fuerte competencia. La mixodiasporia se ha demostrado que reduce la remoción de semillas en ambientes áridos y semiáridos, pero ni el papel de este mecanismo en el conjunto de las comunidades vegetales de las zonas erosionadas, ni su carácter adaptativo ha sido estudiado.

Una semilla pegada al suelo no sólo reduce el movimiento por erosión, sino que también impide la recolección masiva de semillas por animales. Las hormigas granívoras son los principales depredadores de semillas en pastos, estepas y matorrales de las zonas semiáridas de la cuenca mediterránea, sobre todo durante la primavera y el verano. La recolección de semillas por animales granívoros se redujo significativamente cuando las semillas estaban pegadas al suelo o la masa de la semilla fue incrementada por las partículas de suelo adheridas a la capa de mucílago, lo que al mismo tiempo provocó un efecto de camuflaje para la semilla. Comparando la depredación de frutas sueltas y secas con las semillas pegadas al suelo de *Artemisia monosperma*, las sueltas se recogieron mucho más fácil y más rápido que las pegadas. Para las semillas de *Salvia columbariae* se encontraron resultados similares, en los que la recolección de semillas por hormigas granívoras se redujo significativamente en un 94 % para las semillas camufladas (semillas con mucílago secretado y luego recubiertas de arena). Sin embargo, esos experimentos se realizaron en tiempos muy cortos y todavía no se han realizado estudios de larga duración, a lo largo de varias semanas o durante el período completo de germinación de la semilla.

Las funciones del mucílago en semillas seguramente no puedan reducirse a una sola, y las ideas contradictorias sobre su papel ecológico pueden depender de las especies de plantas estudiadas. Sin embargo, en algunos grupos de plantas la influencia del mucílago en la capacidad de germinación de las semillas podría ser excluida si la semilla también presenta otras características, como una larga dormancia. Hay muchas especies en las que la dormancia fisiológica es la principal razón para el aplazamiento de la germinación, causada por la falta de permeabilidad de la testa o el pericarpio de las semillas o del fruto al agua. Antes de que estas semillas puedan germinar, la capa impermeable tiene que ser rota o al menos abierta para que el agua y los gases puedan llegar al embrión. Esto se ha demostrado en especies con dormancia fisiológica, en las cuales las semillas empezaron a germinar por el daño mecánico o químico de su cubierta. La dormancia física está presente en al menos 15 familias de angiospermas, entre otras en las Anacardiaceae, Bombaceae, Cistaceae, Fabaceae, Malvaceae y Rhmnaceae. Por lo tanto, en especies de estas familias, la secreción de mucílago de la semilla parece no ser relevante para la germinación, sino que parece más probable que cumpla una función relacionada con la dispersión de las semillas.

Hipótesis

En el presente trabajo queremos probar críticamente la hipótesis de que la secreción de mucílago en semillas de plantas de simiente dura puede funcionar como un mecanismo antitelecórico, pegando las semillas al suelo y evitando su remoción. Nuestra hipótesis es que la secreción de mucílago de semilla debe considerarse una adaptación de las plantas que habitan en ecosistemas secos y abiertos, como matorral semiárido y mediterráneo, a los riesgos que imponen las condiciones de erosión y la depredación de semillas por hormigas granívoras.

También tenemos como objetivo estudiar la historia evolutiva de la secreción de mucílago de semilla. Dado que las evidencias sobre el carácter ancestral de la secreción de mucílago de semilla son contradictorias, nuestro propósito es analizarlo en toda una familia. Elegimos la familia de las Cistaceae porque es un componente importante de la flora mediterránea, con una parte de esta familia evolucionada recientemente. Además, la presencia del carácter del mucílago en las semillas es muy heterogénea a lo largo de la familia y las semillas presentan dormancia física.

A lo largo de la tesis aplicamos diversos enfoques a las pruebas de estas hipótesis:

1) Probamos si la secreción de mucílago de las semillas impide o dificulta que sean removidas por procesos erosivos (Capítulo 1). Comprobamos experimentalmente si un incremento en la cantidad de mucílago secretado por las semillas reduce sus posibilidades de ser removidas por el impacto de las gotas de lluvia y por la escorrentía. Del mismo modo, también se evaluó el efecto del ambiente materno en la cantidad de mucílago secretado por las semillas, mediante la comparación del mucílago de semillas recogidas de individuos de plantas que viven en ambientes erosivos y no erosivos.

2) Comprobamos la hipótesis de Ellner y Shmida de que el mecanismo de dispersión antitelecórico en ambientes semiáridos no es una adaptación de las plantas al aumento de la aridez o a las limitaciones que impone, sino más bien un efecto secundario de otras fuerzas como la erosión o la depredación de semillas (Capítulo 2). Para comprobarlo se analizaron, en el ámbito de la comunidad vegetal, el efecto cruzado del aumento de aridez y del aumento de erosión en la proporción de especies de plantas con semillas mucilaginosas.

3) Para comprobar si la secreción de mucílago reduce la recolección de semillas por hormigas granívoras, hemos comparado la proporción de semillas recogidas por hormigas entre las semillas pegadas a la tierra con su propio mucílago y otras semillas de la misma especie sin mucílago secretado (Capítulo 3).

4) Para probar si la secreción de mucílago de las semillas del género Fumana es un carácter derivado, hemos reconstruido un árbol filogenético molecular del género usando dos marcadores de plástidos (*matK* ,*trnTL)* y un marcador nuclear (ITS), en el que fueron analizados la aparición de mucílago en semillas y otros caracteres morfológicos (Capítulo 4) .

5) Para estudiar la historia de la evolución del carácter de mucílago en semilla en la familia de la Cistaceae, trazamos la aparición del mucílago a lo largo del árbol filogenético ya construido por otros autores. Para probar la hipótesis de que la secreción del mucílago en esta familia está relacionada con hábitats estresantes, relacionamos en condiciones experimentales el carácter del mucílago y la capacidad de las plantas para colonizar hábitats muy competitivos (Capítulo 5).

Discusión General

Nuestro estudio sobre la secreción de mucílago en semillas mostró claramente que la mixodiasporia funciona como mecanismo antitelecorico en varias especies de Cistaceae. Mostramos que el mucílago pegó efectivamente las semillas al suelo, lo que impidió el movimiento bajo condiciones erosivas simuladas (impacto de gota de lluvia y escorrentía) y redujo la recolección de semillas por hormigas granívoras. En cuanto a la historia evolutiva del mucílago en la familia de la Cistaceae, encontramos que el carácter ancestral es la presencia de mucílago, mientras que la pérdida de mucílago es un carácter derivado. El mismo patrón se observó cuando se analizó el género *Fumana*, en el que una fuerte secreción de mucílago se reveló como el carácter ancestral, con un cambio hacia un estado débil o incluso ausente mucílago en algunas de las especies más recientes.

Nuestros resultados muestran que la secreción de mucílago funciona eficientemente como un mecanismo antitelecórico, como ha sido propuesto por varios autores, ya que las semillas de las especies estudiadas que han sido pegadas al suelo con su propio mucílago sobreviven más tiempo a la depredación por hormigas granívoras, al arrastre por escorrentía y a la erosión que las semillas sin mucílago.

En relación con el papel de la secreción de mucílago en la restricción de la depredación de semillas por hormigas, nuestro trabajo va más allá en el marco temporal que los estudios experimentales previos, que recogieron sus efectos sólo durante unos días, lo que podría ser tiempo insuficiente para garantizar que el mecanismo funcione hasta que las semillas puedan germinar. En la *F. ericoides*, las semillas germinan entre 3-24 meses después de ser dispersadas y en la *F. thymifolia* normalmente entre 4-5 meses (50% germinadas), por lo tanto están expuestas a recolección en la temporada en que las hormigas granívoras tienen su máxima actividad, desde la primavera hasta finales del verano. En el presente estudio, las semillas de *F. ericoides* pegadas a la tierra con su propio mucílago mantuvieron un 71 % de probabilidad de supervivencia, incluso después de 3 meses (91 días) y *F. thymifolia* 68 % después de 2,5 meses (84 días), por lo tanto, casi el doble de posibilidades de supervivencia para esas semillas .

Algunos autores propusieron que la antitelecoria no debe considerarse como una adaptación a la aridez por sí misma o por las restricciones que impone la falta de agua a las semillas. Sino que se debería considerar como un efecto secundario de la consecuencia de esta aridez, es decir, la posibilidad de que las semillas sean recolectadas por los animales o ser removidas por los procesos de erosión en tales hábitats abiertos. La frecuencia de plantas con mecanismo antitelecórico, como la mixospermia, sería entonces favorecida en las comunidades de plantas afectadas por la erosión del suelo, pero no se vería afectado por el aumento de la aridez. En consecuencia, encontramos una mayor frecuencia de especies con recursos antitelecóricos en las comunidades vegetales de las zonas afectadas por la presión de la erosión, en comparación con las comunidades de las zonas planas, sin erosión del suelo, pero la frecuencia no fue influenciado por el aumento de la aridez. Por el contrario, la proporción de especies con recursos atelecóricos (con ausencia del mecanismo de dispersión) aumentó ligeramente con la sequedad, pero no fue influenciado por la erosión del suelo. Estos resultados están de acuerdo con otros que apoyan la hipótesis de que los mecanismos de anclaje en diásporas desempeñan un papel en la conformación/composición de las especies en las comunidades afectadas por una intensa erosión del suelo.

Sin embargo, la secreción de mucílago como mecanismo antitelecórico no sólo podría ser un efecto secundario sino un mecanismo de adaptación per se a la escorrentía en el suelo. Pusimos a prueba la respuesta de adaptación de semillas mixospérmicas a la erosión del suelo esperando encontrar dos supuestos hechos realidad: en primer lugar, que existen dentro de poblaciones y entre poblaciones variación de esta capacidad, y que esta variación se debe relacionar con la presión de la erosión del suelo en el que viven las plantas y segundo, que una mayor cantidad de mucílago podría estar relacionada con una mayor adherencia al suelo y una mayor resistencia de las semillas a ser removidos por la lluvia (impacto de gota de lluvia y el escorrentía de agua).

De acuerdo con nuestra hipótesis, hemos detectado una variación individual de mucílago de semillas entre los individuos dentro de la población y entre las poblaciones. Se encontró una mayor cantidad de mucílago en semillas recogidas de individuos que habían crecido bajo condiciones de alta presión erosiva del suelo. Sin embargo, eso resultados son sólo válidos para *F. ericifolia* pero no para *H. violaceum*, para la que las diferencias no fueron significativas. Apoyando nuestra hipótesis también encontramos que una mayor cantidad de mucílago de semilla se tradujo en una adherencia de semillas, más fuerte y por más tiempo, bajo impacto de gotas de agua en una de las especies estudiadas (*F. ericifolia*) pero no en la otra especie (*H. violaceum*).

En consecuencia, el entorno donde vive la madre de la planta tiene un impacto crítico en el éxito del establecimiento de plántulas de F*. ericifolia*, como ocurre con los ecosistemas áridos y semiáridos que se caracterizan por una escasa vegetación, que a menudo está dispuesta como un mosaico con parches con vegetación densa y parches de suelo vacío que está bajo la influencia de la escorrentía de agua y la erosión del suelo.

Nuestros resultados indican que la secreción de mucílago puede ser una respuesta adaptativa, que parece ser dependiente de la especie. Sin embargo, que esta respuesta dependa de la especie podría estar relacionado con las diferencias en el tipo de mucílago de semilla entre las especies estudiadas. Hasta ahora poca atención se ha puesto en las diferencias estructurales del mucílago en las semillas dentro de una familia de plantas. Aquí, encontramos que la *H. violaceum* tiene un mucílago del tipo pectina mientras que la *F. ericifolia* tiene un mucílago celulósico. Los dos tipos se comportaron de manera diferente bajo la escorrentía y las condiciones del impacto de la gota de agua. Las semillas con mucílago celulósico de *F. ericifolia* mostraron una adhesión más fuerte en los experimentos de escorrentía (100 % de semillas permanecieron pegadas durante el experimento), que las semillas con mucílago pectínico de *H. violaceum* (60 % de las semillas se quedaron pegadas), lo que podría ser un resultado de la fuerza adicional que se supone que añaden los hilos celulósicos al mucílago. Del mismo modo encontramos una relación positiva entre la secreción de mucílago de las semillas de *F. ericifolia* y su resistencia al impacto de gota de agua, mientras que en semillas de *H. violaceum* no se encontró relación evidente.

Al analizar las relaciones filogenéticas y la aparición de mucílago en la familia de las Cistaceae, se encontró que en las semillas el carácter ancestral más probable es la presencia de mucílago, con una tendencia a la pérdida del mismo hacia los casos más recientemente separados (*Cistus, Halimium*). Como se supone que las especies mixospérmicas de esta familia se han adaptado a los procesos de erosión del suelo, podemos predecir que las estrategias del ciclo de vida de esas plantas para colonizar en esas condiciones ambientales erosivas deben diferir fuertemente de la de las especies no mixospérmicas. La erosión del suelo es un proceso geomorfológico que aumenta tanto la tensión como la frecuencia de las perturbaciones que afectan a las plantas y por lo tanto tiende a reducir la cubierta vegetal y la diversidad de especies. Las estrategias del ciclo de vida para hacer frente a esta condición ambiental deben entonces enfocarse en proporcionar semillas con suficientes recursos para establecerse y sobrevivir en condiciones tan pobres. Por el contrario, las especies con semillas sin secreción de mucílago pueden ser arrastradas con frecuencia cuesta abajo, donde tendrán que competir con otros congéneres y otras especies de plantas. Al centrarse en las estrategias del ciclo de vida, se encontró una relación significativa entre la pérdida de mucílago en semillas y los rasgos relacionados con la competitividad, como la tasa de crecimiento relativo (RGR), la longevidad de las semillas y un tamaño más pequeño de la semilla. Esto demuestra la ventaja que tienen las plantas con semillas sin mucílago para establecerse y sobrevivir bajo condiciones de mayor competitividad o bajo una mayor frecuencia de perturbaciones ambientales que otras especies de Cistaceae con semillas mucilaginosas. Esto es coherente con la hipótesis de Grime, según la cual los hábitats no perturbados y ricos en nutrientes deberían tener más especies con altas capacidades competitivas, en comparación con los hábitats perturbados y estresantes, como los entornos erosivos en el presente caso.

La historia filogenética de la familia de la Cistaceae emerge desde el Mioceno hasta el Pleistoceno, y los cambios climáticos y ecológicos parecen haber provocado diversos cambios en las características de las plantas, lo que indica que presiones similares podrían haber provocado cambios en el mucílago de semillas y en los rasgos competitivos. Analizando el carácter ancestral del género *Fumana*, se encontró una tendencia similar: que el carácter ancestral más probable es una fuerte secreción de mucílago con una tendencia hacia su pérdida en un grupo de especies más recientemente separadas. Sin embargo, tres de las cuatro especies del género Fumana con menor cantidad de mucílago se encuentran con frecuencia en entornos montañosos donde se esperaba una gran cantidad de mucílago, debido a la presión de la erosión del suelo y las ventajas de un mecanismo antitelecórico. Curiosamente, el cambio hacia una pérdida de mucílago de semillas en estas especies está conectado con un cambio en las capacidades de dispersión de la semilla en general, en las que se cambia de la dispersión por semillas a una dispersión del fruto entero, quedando la semilla retenida en el interior de la cápsula. El entorno de estas especies es muy rocoso, por lo que sospechamos que la dispersión de semillas dentro de la cápsula les confiere más posibilidades de ser captadas en una grieta rocosa que a una sola semilla mucilaginosa, que podría quedar pegada a las piedras donde el establecimiento de plántulas sería imposible. En este caso, la pérdida de mucílago de semilla es provocada por otros factores que en el caso de la familia de la Cistaceae.

Concluyendo, la familia Cistaceae ofrece un caso interesante para estudiar el origen y la función de mucílago de semillas desde el punto de vista ecológico y evolutivo. Nuestros resultados ofrecen evidencia sobre el carácter ancestral de esta característica y las fuerzas ambientales que le dieron forma. También encontramos apoyo para nuestra hipótesis de que la secreción de mucílago en las semillas de las especies de esta familia puede estar relacionada con una colonización exitosa de hábitats abiertos y frecuentemente erosionados. Encontramos que existe variabilidad fenotípica en la cantidad de mucílago secretada por las semillas en algunas especies y que esta variabilidad está relacionada con la capacidad de estas semillas para resistir a las fuerzas erosivas. Sin embargo, la heredabilidad de este rasgo permanece sin testar. Se necesita más investigación para confirmar esta tendencia en otras familias de plantas que viven en condiciones semiáridas, y también para profundizar en el conocimiento de la relación entre los diferentes tipos de mucílago, su historia evolutiva y sus funciones ecológicas.

Conclusiones

I. La secreción de mucílago reduce la remoción de semillas producida por los procesos erosivos que se originan en condiciones naturales. Estos procesos pueden ser: desprendimiento por gota de lluvia y remoción por la escorrentía de agua.

II. El valor adaptativo de la secreción de mucílago depende de la especie, así encontramos una relación entre la cantidad de mucílago de semilla y la fuerza de la adhesión en el caso del impacto de gota de lluvia para *Fumana ericifolia*, pero no para *Helianthemum violaceum*.

III. El valor adaptativo de las secreciones de mucílago parece depender del mecanismo, así encontramos que la pérdida de semillas por impacto de gota de lluvia fue proporcional a la cantidad secretada de mucílago de semilla, pero fue así no para el proceso de escorrentía de agua.

IV. La cantidad de mucílago de semillas en algunas especies está influenciada por las condiciones en las que la planta madre vive, ya que se detectó una relación positiva entre la erosión que la planta madre experimenta y la cantidad de mucílago secretada por las semillas de estas plantas en *Fumana ericifolia*. A pesar de ello, el individuo y no la población de la que se recogió la semilla tuvieron la mayor influencia sobre la variación en la cantidad de mucílago producido.

V. Los resultados indican que los mecanismos antitelecóricos, como la mixodiasporia, no se adaptan a los ambientes áridos porque no se ha detectado que un aumento en la aridez cause un aumento en la frecuencia de su aparición. Por otra parte, los mecanismos antitelecóricos parecen ser favorecidos por la escorrentía superficial, como se predijo por Ellner y Shmida (1981), ya que se detectó una mayor frecuencia de especies con mecanismos antitelecóricos en áreas con condiciones erosivas que en las zonas sin condiciones erosivas.

VI. Los mecanismos de anclaje de las diásporas influyen fuertemente en la composición de las comunidades de especies afectadas por la erosión, así que la erosión intensa del suelo puede dar forma a la composición de las comunidades de especies.

VII. Las especies estudiadas fueron extensivamente depredadas por diversas hormigas granívoras en el área de estudio, ya que se encontró que flores, cálices, cápsulas y semillas fueron muy depredados durante el período de tiempo estudiado. El mucílago de semillas reduce significativamente la depredación por hormigas granívoras. Al comparar las semillas que fueron pegados a la tierra con su propio mucílago previamente secretado y las semillas sin mucílago, las probabilidades de supervivencia de las semillas fueron un 54 % más altos para *R. officinalis*, el 58% para *F. ericoides* y el 54 % para F*. thymifolia*. Una tasa mayor de supervivencia de semillas debe aumentar el índice de establecimiento de plántulas de las especies estudiadas.

VIII. Las relaciones filogenéticas de la *Fumana*, basadas en la reconstrucción filogenética a partir de dos marcadores moleculares, confirmaron la monofilia. Los resultados no son compatibles con las divisiones infragenéricas tradicionalmente establecidas, pero confirman la presencia de dos grupos principales de especies.

IX. Los tiempos de divergencia de *Fumana* tuvieron lugar sobre 16.97 Ma atrás (24-10 Ma), con una divergencia de las principales clados entre el Mioceno medio y superior (15.61 a 8.8 Ma).

X. Se realizó una reconstrucción de los estados ancestrales en nueve caracteres morfológicos en el género *Fumana*. Estados ancestrales significativos fueron encontrados en cinco casos (forma y márgenes de la hoja, tricomas glandulares, secreción de mucílago). Esto sugiere una adaptación al medio ambiente en el clima mediterráneo.

XI. La secreción de mucílago en semillas en el género *Fumana* es el estado ancestral más probable, habiendo evolucionado de una fuerte secreción de mucílago a una forma reducida y casi ausente de mucílago en 4 de las 19 especies estudiadas.

XII. La secreción de mucílago en semillas en el género de las Cistaceae parece ser el estado ancestral de este carácter. Además, el mucílago de las semillas parece haberse desarrollado de mucílago pectínico a un mucílago celulósico en el género *Fumana*, y a una pérdida total del mucílago de semillas en el género *Cistus*.

XIII. Se encontraron dos patrones en especies de la familia de las Cistaceae:

1º. Fuerte secreción de mucílago de tipo celulósico, bajos valores de RGR, una reducida longevidad y tamaños grandes de la semilla en los géneros más antiguos (es decir, *Fumana*).

2º. Ausencia de secreción de mucílago, un mayor RGR, semillas con mayor longevidad y un tamaño más pequeño de la semilla en las especies más modernas, es decir en el género *Cistus*.

Introduction

Formation and composition of mucilage

In ripe seeds and fruits (diaspores), the seed coat or the pericarp acts, respectively, as a protection of the embryo against early desiccation and physical or biological damage. The seed coat or pericarp can also act as a regulator of water uptake, releasing seed germination in some species or maintaining seed dormancy by preventing water absorption and gas exchange in others. In a few cases, the seed coat has been reported to function as a reserve tissue during germination. Together to germination, in those species in which the seed or the fruit are the dispersal units, the seed coat or the pericarp can also play a role in seed dispersal by modifying its properties or developing specialized structures (Werker, 1997).

An outstanding modification of the diaspore surface structure in many species of the Angiosperms is the presence of a mucilaginous layer. In these species, when the dry seed coat or the pericarp comes in contact with water, imbibes the outer cell wall completely and release a mucilaginous substance; a phenomenon known as myxospermy. Mucilage is composed of polysaccharides, mostly of pectins, and forms a gel like envelop around the diaspore that holds a considerable amount of water due to its hydrophilic nature. The seed weight as well as the volume increases significantly once the mucilage is released (Deng et al., 2012). Once it dries up, mucilage becomes stiff then gluing the diaspore to the surface on which it settles.

There are differences in mucilage composition depending on the species. However, the main component of the mucilage of the pericarp and seed coat in all species are pectins. Most studies about the components of seed mucilage use seeds of *Arabidopsis thaliana* (Macquet et al., 2007; Moïse et al., 2005; Penfield et al., 2001; Usadel et al., 2004; Western, Skinner and Haughn, 2000), but there are also studies examining mucilage composition from basil's seeds (Ocimum ssp. ;Anjaneyalu, Khan and Tharanathan, 1983; Anjaneyalu, Khan and Tharanathan, 1984; Khan et al., 1987), flax (Linum ssp. ;Fedeniuk and Biliaderis, 1994; Naran, Chen and Carpita, 2008) and plantains (Plantago ssp. ;Guo et al., 2009; Yamada et al., 1986).

Pectins consist of a heterogeneous group of acidic polysaccharides characterized by the presence of galacturonic acid (GalA). To simplify, the pectin backbone comprises 2 key types of polymer: rhamnogalacturonan I (RG I) and homogalacturonan (HG) (Haughn and Western, 2012). When analyzing the different sugars which are contained in the mucilage, significant amounts of neutral sugars, other than rhamnose (Rha), were detected, mainly galactose (Gal) and glucose (Glc), but also fucose (Fuc), arabinose (Ara), xylose (Xyl) and mannose (Man) [34–48% (w/w) of the total neutral and acidic sugars in mucilage] (Penfield et al., 2001; Usadel et al., 2004; Western et al., 2000). The polysaccharides and acidic qualities of mucilage make them very hydrophilic so in the presence of water they hydrate rapidly, thus forming super absorbent hydrogels (Deng et al., 2012; Fahn and Werker, 1972; Frey-Wyssling, 1976; Zwieniecki, Melcher and Holbrook, 2001). After water absorption, the mucilage breaks through the cell wall forming the mucilaginous envelope surrounding the seed.

In addition to pectin, mucilage in some species also contains strands of elementary fibrils of cellulose of different widths embedded in the pectin envelope (Fahn and Werker, 1972; Kreitschitz and Vallès, 2007; Mühlethaler, 1950; Schnepf and Deichgräber, 1983). Mucilage can therefore be distinguished in “true” mucilage in most species consisting almost exclusively of pectins, and “cellulosic” mucilage, which, additionally to pectins, also contains cellulose fibrils. However, in some species the “true” mucilage can contain hemicellulose, as for example occurring in flaxseeds (Linaceae), in which together with the RGI, more than 50% of arabinoxylose was found. So these established definitions should be revised in future studies.

Cellulosic mucilage seems to add an additional strength to the pectin mucilage layer and has been hypothesized that it prevents mucilage of being washed away from the seed coat or fruit pericarp making the mucilage more rigid and thus, resulting in an enhanced adhesion of the mucilage to the diaspore (Grubert, 1974; Harpaz-Saad et al., 2011; Sullivan et al., 2011). This structural difference influence the degree of adhesion of diaspores to any surface, therefore helping their transport on the fur or feathers of animals, preventing seed removal by erosion and predation by animals and also helping the root to penetrate into the soil (Gutterman, Witztum and Evenari, 1967).

Cellulosic mucilage has been found in various plant families amongst others in Asteraceae, Brassicaceae, Cistaceae, Euphorbiaceae, Lamiaceae and Polemoniaceae (genus Fumana, Grubert, 1974; Hedge, 1970; Kreitschitz and Vallès, 2007; Schnepf and Deichgräber, 1983; Vaughan and Whitehouse, 1971) while pure pectin mucilage has been reported amongst others for Linaceae, Plantaginaceae, Poaceae and Cistaceae (genus Helianthemum, Fahn and Werker, 1972; Grubert, 1974; Hyde, 1970; Kreitschitz, Tadele and Gola, 2009; Mühlethaler, 1950). Simple staining reactions as assembled by Kreitschitz (2007) help to distinguish between both types of seed mucilage. Ruthenium red as well as safranin provides very strong staining reactions for pectin, in which ruthenium red typically reveals their homogenous structure. Staining with methylene blue or I in KI+H2SO4 confirms the presence of cellulosic strands in the pectin envelope.

Seed mucilage production in *Arabidopsis thaliana* is part of an exceptional differentiation process during which the epidermal cells of the mature ovule grow, rearrange their cytoplasm, synthesize and secrete mucilage, and form a secondary cell wall (Western et al., 2000). After a phase of extensive mucilage production, mucilage is placed between the plasma membrane and the outer periclinal wall of the epidermis cell, resulting in the compression of the protoplast (Windsor et al., 2000). This reduction of the protoplast results in the formation of a volcano-shaped cytoplasmic column that lies under the mucilage pocket. Synthesis of a cellulosic cell wall to surround the cytoplasmic column results in the formation of the volcano-shaped columella (Western et al., 2000; Windsor et al., 2000; Western, 2012).

The mucilage originates from specialized epidermal cells of the seed coat in most species (Fahn and Werker, 1972; Grubert, 1974; Grubert, 1981; Haughn and Western, 2012; Werker, 1997; Western et al., 2000) but can also be present in both, epidermal and sub epidermal cells or only in the latter (Werker, 1997). Furthermore in some species of the Lythraceae, mucilage occurs only in a certain parts of the seed (Panigrahi, 1986), in sacs within the seed coat, as in the testa of Sterculiaceae (Boesewinkel and Bouman, 1984; Corner, 1976) or the mucilage layer is discontinuous as cells with mucilage are scattered between cells without mucilage (Lamiaceae , Witztum, 1978). Also mucilage cells can be present at the tip of hairs, which can be unicellular as in *Ruellia* (Grubert, 1974; Haberlandt, 1965) or multicellular as in *Blepharis ciliaris* or *Blepharis persica* (Gutterman and Witztum, 1977; Gutterman et al., 1967). Furthermore, mucilage is also found in the pericarp of diaspores as in the Asteraceae, Lamiaceae, Piperaceae or Urticaceae (Grubert, 1974; Ridley, 1930; Werker, 1997). Mucilage, when the origin of the mucilage producing tissue varies between the testa and pericarp, can then be classified following Ryding (2001) into “myxodiaspory” as the condition of having mucilaginous diaspores, “myxocarpy” when the carpel or pericarp produces mucilage while “myxospermy” refers to the condition of mucilaginous seeds.

Origin and ancestry of mucilage

Mucilage release after wetting in seeds and fruits is a common feature in many families of Angiosperms. From bibliographical references we found that 1369 species of 102 plant families (Table 1), belonging to 40 different orders have diaspores which secrete mucilage on wetting. Most of these species were found in the plant family of the Asteraceae (197 species), Lamiaceae (158), Acanthaceae (144), Fabaceae (86), Brassicaceae (81), Cistaceae (76), Euphorbiaceae (52), Polemoniaceae (42), Plantaginaceae (32), Malvaceae (30), Podostemaceae (20), Linaceae (19), Rosaceae (18), Araceae (16), Rutaceae (16), Arecaceae (16), Scrophulariaceae (16), Poaceae (15), Solanaceae(15), Violaceae (14), Nyctaginaceae (13) and followed by 89 further plant families which have been found to have at least between 1 and 10 species with mucilaginous seeds or diaspores.

Additional to the bibliographic references, in 2010 I performed at the facilities of the Millennium Seed Bank- Royal Botanic Gardens, Kew, a survey of mucilage secretion by diaspores of 237 species belonging to 95 plant families, and found that 8.4% of these species (20 species), belonging to 9.5% (10) of the tested families secrete mucilage. When adding up the bibliographic information and my own survey, I found that a total of 108 Angiosperm families have diaspores that secrete mucilage on wetting.

Many plant families have seeds without mucilage secretion. Negative annotations were obtained by testing at least two species of as many genera as possible or detailed description in literature. Furthermore certain characteristics were classified to be incompatible to the production of seed mucilage (for example very big or winged seeds). The following list represents families for which no mucilage in seeds could be found:

Alseuosmiaceae, Apocynaceae , Araliaceae, Aceraceae, Begoniaceae, Betulaceae, Bignoniaceae, Bixaceae, Burseraceae, Buxaceae, Canellaceae, Cannabaceae,Calycanthaceae, Calyceraceae, Chrysobalanaceae, Clethraceae, Commelinaceae, Coriariaceae, Cornaceae, Crassulaceae, Crossosomataceae, Cunoniaceae, Cyrillaceae, Daphniphyllaceae, Diapensiaceae, Dipterocarpaceae Droseraceae, Elaeagnaceae, Elaeocarpaceae, Elatinaceae, EleagnaceaeEscalloniaceae, Eucommiaceae, Fouquieriaceae, Gentianaceae, Geraniaceae, Griseliniaceae, Gyrostemonaceae, Haemodoraceae, Haloragaceae, Hamamelidaceae, Hernandiaceae, Hydrangeaceae, Hippocastaneaceae, Icacinaceae, Liliaceae, Loasaceae, Magnoliaceae, Malpighiaceae, Marcgraviaceae, Melastomataceae, Meliaceae, Menispermaceae, Menyanthaceae, Molluginaceae, Monimiaceae, Myrtaceae, Nelumbonaceae, Ochnaceae, Oleaceae, Oxalidacea, Papaveraceae, Parnassiaceae, Passifloraceae, Phrymaceae, Pittosporaceae, Plumbaginaceae, Polygalceae, Polygonaceae, Proteaceae, Resedaceae, Salicaceae, Sapotaceae, Sarraceniaceae, Staphyleaceae, Stylidiaceae, Styracaceae, Surianaceae, Tamaricaceae, Theaceae, Theophrastaceae, Thymelaeaceae, Tiliaceae, Trapaceae, Ulmaceae, Vahliaceae, Verbenaceae, Vitaceae, Winteraceae. Resedacea.

In a recent study, Yang et al. (2012b) proposed that seed mucilage must be an evolutionary advanced trait because they found a relation between the ordinal phylogenetic position of plant families and the frequency of myxodiasporic taxa they comprise. Furthermore, when they inspected the habitat of the species of the families with myxodiasporic species, they found a higher frequency of taxa living in dry habitats in phylogenetically advanced families (e.g. Asteraceae, Brassicaceae and Poaceae) than in basal ones (e.g. Nymphaeales and Magnolids in moist habitats). However, in their analysis Yang and colleagues (2012b) only account for the absolute numbers of myxodiasporic taxa, not for their relative numbers. There are families with a very large amount of species in contrast to families with a low number of species, thus it is much more likely to find references for myxodiaspory in those families. Also, in many cases, the larger families are usually those in which more research has been conducted resulting in a higher rate of mucilage discovery. Nevertheless, we propose, that, since mucilage secretion in seeds or fruits has been found in many plant species, families and orders all along the angiosperm family tree, and also there is paleontological evidence that seed mucilage existed as early as in the Middle Eocene (Smith and Stockey, 2003), the character of seed mucilage could be considered as ancestral. The character might have been lost in some branches of the clade as it has been conserved in others. This hypothesis could be proven at different levels of the angiosperm phylogenetic tree, comparing orders and families, or comparing genera and species within families. Furthermore, the ancestral character state of myxodiaspory in a certain group of plants could be tested, as well as a differentiation of the type of mucilage could be made, distinguishing between pectic and cellulosic mucilage. Even though the result of an ancestral analysis performed at a smaller level might not reveal the real origin of mucilage in the Angiosperm phylogenetic, it still opens many opportunities to discuss the actual function and a hypothetical former function of seed mucilage secretion in this plant family or genus.

Table 1: Bibliographic references for plant families in which species with seed or fruit mucilage secretion were found.

Family	*Reference*	*Family*	*Reference*
Actinidiaceae	(1)	**Acanthaceae**	(2)(3)(4)(5)(6)
Acoraceae	(7)	**Adoxaceae**	(4)(8)
Aizoaceae	(4)	**Amaranthaceae**	(4)
Amaryllidaceae	(4)	**Anacardiaceae**	(4)
Annonaceae	(4)	**Apiaceae**	(4)
Apocynaceae	(4)	**Aquifoliaceae**	(4)
Araceae	(7)(4)	**Arecaceae**	(4)
Aristolochiaceae	(1)	**Asteraceae**	(9)(10)(11)(4)(12)(13)(14)(15)(16)
Balsaminaceae	(10)(4)	**Berberidaceae**	(4)
Bignoniaceae	(4)	**Bixaceae**	(4)
Bombacaceae	(17)	**Boraginaceae**	(4)
Brassicaceae	(10)(18)(19)(20)(21)(4)(12)(22)(23)(24)(25)(8)(26)(27)(28)(29)	**Burseraceae**	(4)
Cactaceae	(4)	**Campanulaceae**	(4)(1)
Capparaceae	(4)	**Caprifoliaceae**	(4)(8)
Caricaceae	(4)	**Caryophyllaceae**	(4)
Casuarinaceae	(1)	**Celastraceae**	(4)
Cistaceae	(4)(30)(12)(31)(32)(8)	**Combretaceae**	(4)
Connaraceae	(4)	**Convolvulaceae**	(4)
Crassulaceae	(1)	**Cucurbitaceae**	(4)
Cyperaceae	(4)	**Dilleniaceae**	(4)
Dioscoreaceae	(4)	**Dipsacaceae**	(4)
Ebenaceae	(4)	**Ericaceae**	(4)
Euphorbiaceae	(33)(34)(4)(35)(36)(8)(28)	**Fabaceae**	(4)(37)
Frankeniaceae	(34)	**Garryaceae**	(1)
Geraniaceae	(37)	**Gesneriaceae**	(4)
Goodeniaceae	(4)	**Hydrocharitaceae**	(7)(4)(37)
Hydrostachyaceae	(4)	**Iridaceae**	(4)
Juncaceae	(4)(37)(8)	**Lamiaceae**	(10)(38)(3)(18)(4)(39)(14)(40)(8)(28)
Lauraceae	(41)(4)	**Lentibulariaceae**	(4)
Liliaceae	(4)	**Limnanthaceae**	(4)
Linaceae	(10)(18)(42)(43)(4)(23)(14)(44)(37)(8)(26)	**Loganiaceae**	(4)(1)
Loranthaceae	(45)(4)(12)	**Lythraceae**	(4)(37)
Malvaceae	(4)	**Marantaceae**	(4)
Martyniaceae	(4)	**Menispermaceae**	(4)
Mimosaceae	(12)	**Moraceae**	(4)

Musaceae	(4)	**Myrtaceae**	(4)
Nyctaginaceae	(7)(4)(34)	**Nymphaeaceae**	(4)
Oleaceae	(4)	**Onagraceae**	(4)(28)
Orchidaceae	(4)	**Oxalidaceae**	(4)
Palemoniaceae	(8)	**Pandanaceae**	(1)
Papaveraceae	(4)	**Passifloraceae**	(4)
Piperaceae	(37)	**Plantaginaceae**	(4)(12)(46)(23)(14)(47)(37)(8)(26)(28)
Poaceae	(4)(48)(14)	**Podostemaceae**	(37)(4)
Polemoniaceae	(49)(34)(4)(50)(37)	**Ranunculaceae**	(4)(1)
Rhamnaceae	(4)	**Rosaceae**	(18)(4)
Rubiaceae	(4)	**Rutaceae**	(4)
Salicaceae	(4)	**Salpindaceae**	(4)
Salvadoraceae	(4)	**Sapindaceae**	(4)
Sapotaceae	(4)	**Saxifragaceae**	(33)(4)
Scrophulariaceae	(4)(37)(8)	**Simaroubaceae**	(4)
Solanaceae	(4)(8)	**Sterculiaceae**	(51)(37)
Theophrastaceae	(4)	**Urticaceae**	(4)(37)(8)
Verbenaceae	(8)(4)	**Violaceae**	(10)(8)
Vitaceae	(4)	**Zygophyllaceae**	(4)(4)(37)

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Function of mucilage

Several different functions have been proposed for myxodiaspory which can be grouped under two main hypotheses. One related to seed germination and the other one related to seed dispersal (Cerdà and García-Fayos, 1997; García-Fayos and Cerdà, 1997; Grubert, 1974; Gutterman and Shem-Tov, 1997a; Huang, Gutterman and Osborne, 2004; Ridley, 1930; Ryding, 2001; Van der Pijl, 1972; Witztum, Gutterman and Evenari, 1969; Zaady, Gutterman and Boeken, 1997).

As mucilage absorbs rapidly a big amount of water and retains it during a certain time period, it has been hypothesized that mucilage should serve as a way to absorb and store water for germination (Fahn and Werker, 1972; Gutterman and Shem-Tov, 1997b; Kreitschitz et al., 2009; Sun et al., 2012; WenNi et al., 2011; Young and Evans, 1973). This has been studied by Gat-Tilman (1995), who associates the large mucilaginous envelope of the *Carrichtera annua* seeds which the possible ability to "sense" water and regulate germination. Harper (1966) justify this capability of the mucilage to germination to the facilitation of water diffusion from the substrate to the seed by increasing the number of pathways and its surface area. It is hypothesized that this enlarged area of contact between the diaspore and the soil furthermore minimizes water loss of the seed (Evans, Young and Hawkes, 1979; Gutterman et al., 1967; Hadas, 1982; Mott, 1974; Witztum et al., 1969). A general accelerated germination by the presence of the mucilage envelope has been described for *Anastatica hierochuntica* (Friedman and

Stein, 1980; Gutterman and Shem-Tov, 1997b). Furthermore, mucilaginous seeds of *A. sphaerocephala* were found to show less sensitivity to increasing osmotic potential (PEG) and salinity (NaCl) stress than demucilaged seeds (Yang et al., 2011).

Contrarily, several authors also found that the presence of mucilage inhibited the germination in some other species (Atwater, 1980; Edwards, 1968; Fitch, Walck and Hidayati, 2007; Heydecker and Orphanos, 1968; Witztum et al., 1969). Excess water in the surrounding of seeds of *Blepharis persica and Spinacia oleracea* was found to prevent germination (Heydecker and Orphanos, 1968; Witztum et al., 1969). It was interpreted as an evidence of shortage of oxygen, since in these species seed germination could be enhanced strongly by the removal of the mucilage coat or by increasing oxygen concentration in the surrounding water (Heydecker and Orphanos, 1968; Witztum et al., 1969). Likewise, the increase in mucilage thickness in seeds of *Lesquerella perforata* and *L. stonensis* (Brassicaceae) correlated with a reduction in germination rate (Fitch et al., 2007). Witztum (1969) and Gutterman (1996) interpreted this phenomenon as a mechanism of preventing seed germination in those arid habitats where the first rain predictably does not provide enough water for a successful development and therefore may not be the best opportunity for plant establishment.

Seed mucilage not only secretes under very humid conditions but can also be secreted under light rain and even under night dew, and also it can be rehydrated after drying. This might not trigger germination but can promote other processes in the embryo, such as repair and restoration of the DNA (Huang et al., 2008; Osborne, Sharon and Ben-Ishai, 1980). Seeds deposited on the soil surface under desert conditions can undergo strong insolation. It has therefore been discussed that seed mucilage improves seed survival under strong climatic conditions as the retained water enhances DNA repair of the embryo of the seed. In this context, the DNA of seeds of two *Artemisa* species were artificially damaged via radiation and the influence of the mucilage envelope in repairing the DNA analyzed (Huang et al., 2008). They found that seeds with intact mucilage coat showed DNA restoration while demucilaged seeds did not (Huang et al., 2008).

Once germinated, very young seedlings are susceptible to many hazards such as desiccation (Miles, 1973), pathogens (Augspurger, 1984) or competition from existing vegetation (Aguilera and Lauenroth, 1993). There are diverse authors who proposed that mucilage in seeds influence seedling establishment positively, as for example Huang (2004), who states that the presence of the mucilage envelope considerably increase the endurance of the seedling of *Artemisia spaerocephala* and *A. monosperma* under desert environments. Garwood (1985) found that in fruits of *Cavanillesia platanifolia* (Bombacaceae), a copious mucilage layer was necessary for a successful expansion of the cotyledons and later seedling growth when watering was infrequent. Additionally, mucilage reduced the degree of seedling wilting thus increasing the survival probabilities of the seedling under drought conditions (Garwood, 1985).

It has also been suggested that some organic compounds present in the mucilaginous envelop of the seed can be used by the embryo for its development (Huang and Gutterman, 1999), since seedlings from seeds with intact mucilage layer in *Artemisia monosperma* had greater vigor than those without it. Yang (2012a) shows that polysaccharides (or its derivatives) of seed mucilage are absorbed by roots and subsequently transported to shoots of seedlings, which provides direct evidence for the absorption of mucilage-derived molecules in early seedling growth. Also mucilage as nutrient reservoir has been proposed for *Capsella bursa-pastoris*, hypothesizing that it attracts soil organism, contains proteases and absorbs amino acids (Barber, 1978).

The functions of myxodiaspory have also been frequently discussed in relation to seed dispersal as the hydrated mucilage coat is extremely sticky, and, once it dries up, works effectively as glue. Two main roles have been recognized for it, first, regarding to the adhesion of the diaspore to the fur and feathers of animals, functioning as a dispersal mechanism (telechory), and second, regarding to its power of gluing the seed to the ground, thus working as anti-dispersal mechanism (antitelechory).

The first role claimed for the sticky character of mucilage is its relation to the long distance dispersal ability of seeds (Grubert, 1974; Ridley, 1930; Ryding, 2001; Swarbrick, 1971; Van der Pijl, 1972). *Euphorbia* species in Hawaii have been reported to be dispersed between islands attached to the bird’s wings caused by the mucilage secretion of their seeds (Baiges, Espadaler and Blanché, 1991; Carlquist, 1966). Furthermore, seeds of *Anastatica hierochuntica* in the Negev Desert are eaten by birds (*Phasianidae*) but at the same time they are likely to adhere to the bird's body and then dispersed to large distances (Friedman and Stein, 1980). According to Weberling (1968) the long distance dispersal of certain Polemoniaceae (*Glieae* and *Polemoniae*) can be interpreted as the result of being transported glued to migrant animals. When mucilaginous fruits and seeds are being consumed by predators, not only can seeds stick accidentally to the animal and be dispersed, but it has also been suggested that the mucilage that covered the seed or fruit provides lubrication for passage through the digestive tract of animals. The germinability of eaten seeds is inversely related to the time they the pass into the digestive track of animals (Verdú and Traveset, 2004) and then early dropping avoids seed damage at the same time that it assures long distance dispersal. This has been described for seeds of *Cecropia*, whose fruits are a main component in the bat diet and whose mucilage cells of the seeds are often found intact in their feces (Lobova et al., 2003). Feeding on seeds that are able to secrete mucilage has also been observed in chukar partridges (*Alectoris gracea*), which consumes seed of yellow flowered pepperweeds (*Lepidium perfoliatum*), tumble-mustard (*Sisymbrium altissimum*) and tansymustard (*Descurainia pinnata*; Savage, Young and Evans, 1969).

Seed mucilage can furthermore work effectively as antitelechoric mechanism. Van Rheede van Oudtshoorn and van Rooyen (1999) describe diverse forms of antitelechoric mechanisms for plants inhabiting dry habitats but they found only two mechanisms in which the seed itself is actually restricting their secondary dispersal in space due to soil adhering mechanisms (myxospermy and trypanospermy; Van Rheede van Oudtshoorn and Van Rooyen, 1999). Trypanospermic diaspores have the ability to bore their diaspores into the soil because drilling mechanisms of the awn, pappus or other seed or fruit coat structures (Stamp, 1984) while myxospermic diaspores glue them to the ground (Grubert, 1974).

Antitelechoric mechanisms have been normally related to the climatic conditions where plants live and have more frequently been reported in arid and semiarid environments than in mesic habitats (Ellner and Shmida, 1981; Jenny and Halfmann, 1993; Ryding, 2001; Vanrooyen, Theron and Grobbelaar, 1990). The theory of the mother site has been established (Zohary, 1937), hypothesizing that in arid and semiarid habitats the vicinity of the mother plant usually has favorable and appropriate conditions for seedling growth. It has been argued that long distance dispersal for species living in those environments carries very little advantage as favorable conditions are close to the mother plant (mother-site) and significant variations in the climate and biotic environment are usually found farther away than the dispersal distance that the seed can achieve (Ellner and Shmida, 1981). In these circumstances, mucilage secretion adhering the seed on crusted soil has been proven to favour establishment in *Helianthemum squamatum*, in which the proximity to a seed source is the main predictor for seedling emergence (Escudero et al., 1999).

Arid and semiarid environments present diverse difficulties for plant establishment. Soil erosion occurs frequently on steep slopes (Poesen and Hooke, 1997) and intense rainfalls are often concentrated into a small number of intense events in Mediterranean and semiarid environments (Rodriguez-Iturbe and Porporato, 2004; Thompson, 2005). Seeds deposited after dispersal on the soil surface of steeped slopes are then at a risk of being removed downslope with runoff water towards the lower parts of the slopes or in the valley bottoms where they can get buried (García-Fayos et al., 1995) or be affected by a stronger competition of seedlings than on the upper or medium parts of the slopes (Cantón et al., 2004). Therefore, on semiarid slopes the removal of diaspores by soil erosion can be a major difficulty to overcome in low competitive plants and, in consequence, anchoring the diaspore to the ground near the mother plant may be a favourable adaptation to avoid strong competition. Myxodiaspory has been proven to reduce seed removal in semiarid and arid environments (Cerdà and García-Fayos, 2000; Friedman and Orshan, 1975; García-Fayos and Cerdà, 1997; Gutterman, 1990; Hsiao and Chuang, 1981; Lu et al., 2010; Sun et al., 2012) but neither the role of this mechanism in the assembly of plant communities of eroded areas nor its adaptive character has been studied.

Gluing the seed to the ground therefore not only hinders further movement by soil erosion, but additionally prevents massive seed collection by animals (Fuller and Hay, 1983; Gutterman, 2003; Gutterman and Shem-Tov, 1997a; Huang, Gutterman and Hu, 2000; Sun et al., 2012; Yang et al., 2013). Harvester ants are the main seed predators in pastures, steppes and scrublands in the semi-arid areas of the Mediterranean basin, mainly during the late spring and summer (Azcárate et al., 2005; Diaz, 1994; García-Fayos and Gasque, 2006; López-Vila and García-Fayos, 2005). Seed collection by granivorous animals was significantly reduced when seeds were glued to the soil or its seed mass incremented due to adhered soil particle on the mucilage coat, which at the same time provoked a camouflaging effect for the seed (Fuller and Hay, 1983; Gutterman and Shem-Tov, 1997a; Young and Evans, 1973). Comparing the predation of loose, dry fruits to adhered achenes of *Artemisia monosperma*, the loose ones were collected much easier and faster than the glued ones (6 hours vs. 3 days; Huang et al., 2000). Similar results were found for seeds of *Salvia columbariae*, in which seed collection by harvester ants was reduced significantly by 94% for camouflaged seeds (seeds with mucilage secreted and then coated in sand; Fuller and Hay, 1983). However, those studies run for a very short time while long time studies over several weeks or a long germination period of the seed are still lacking.

Mucilage secretion can probably not be reduced to only one single function and conflicting ideas about its ecological role may depend on the plant species studied. However, in some plant groups the influence of mucilage on the germination ability of seeds could be excluded if other seed characteristics, such as long dormancy, also apply. There are many species with physical dormancy in which the primary reason for the delay of germination is the lack of permeability of the seed (or fruit) coat to water. Before these seeds can germinate, the impermeable layer needs to be broken or at least opened so water and gases can pass (Rolston, 1978; Werker, 1980; Werker, 1997); as evidenced since in species with physical dormancy the germination is promoted by the mechanical or chemical damage of the seed coat (Baskin and Baskin, 1998). Physical dormancy is present in at least 15 Angiosperm families, amongst others in Anacardiaceae, Bombaceae, Cistaceae, Fabaceae, Malvaceae and Rhmnaceae (Baskin and Baskin, 1998). In the species of these families, seed mucilage seems therefore to be not very relevant for germination and it would be more likely that mucilage secretion played a function related to seed dispersal.

Hypothesis

In the present work we want to critically test the hypothesis that seed mucilage secretion in plants with hard coated seeds can work as an antitelechoric mechanism, gluing the seeds to the ground and hindering further movement. We assume that it is unlikely that mucilage secretion is related to germination functions in plant species in which the seed coat is extremely hard and whose seeds show physical dormancy or deep physiological dormancy with long periods of stratification before germination (sensu Baskin and Baskin, 1998).

We hypothesize that seed mucilage secretion should be considered an adaptation to the hazards that erosive conditions and seed predation by granivorous ants impose to plants that inhabits open dry habitats, such as semiarid and Mediterranean shrubland. Because of the trade-off that exists between colonizing stressful habitats and colonizing high competitive environments (Grime, 2001), plant adaptations to one of these conditions must decrease the ability of these plants to colonize successfully in the other conditions. Therefore, we predicted that if mucilage secretion characterizes plants that colonize stressful habitats, these plants also must have lower competitive ability than phylogenetic related plants that cannot secrete mucilage.

We also aim to study the evolutionary history of seed mucilage secretion. Since evidences are contradictory about the ancestral character of seed mucilage secretion (see above) we aim to analyse it in a whole family. We choose the family Cistaceae because it is important component in the Mediterranean flora, where a part of this family radiated recently (Guzmán, Lledó and Vargas, 2009; Guzmán and Vargas, 2009), because a heterogeneous occurrence of mucilage character along the family has been found and because their seeds present physical dormancy.

Along the thesis we apply diverse approaches and focus to tests these hypotheses:

1) We test if mucilage secretion by seeds prevents them to be removed by erosive processes (Chapter 1). We approached it experimentally by examining whether an increase in the amount of mucilage secreted by seeds reduces its possibilities of being removed by drop impact and by runoff. Likewise, we also evaluated the consequences of the maternal environment on the amount of mucilage secreted by seeds by comparing

seed mucilage of seeds collected from plant individuals living in contrasted erosive environments.

2) We test the hypothesis of Ellner and Shmida (1984) that in semi-arid environments antitelechoric dispersal mechanism is not an adaptive trait of plants to the increase of climate aridity or its constraints, but rather a side-effect to other forces such as erosion or seed predation (Chapter 2). To test it, we analyzed, at the plant community level, the joint effect of increasing aridity and plant erosion on the proportion of plant species with mucilaginous seeds.

3) To test the effect that mucilage secretion has on reducing seed collection by granivorous ants, we compared the ratio of seed removal by ants between seeds glued to the ground with its own mucilage and seeds of the same species without mucilage secreted (Chapter 3).

4) To test if mucilage secretion by seeds in the genus *Fumana* is a derived character state, we reconstructed the molecular phylogeny of the genus based on two plastid and one nuclear marker (*matK*, *trnTL* and ITS) onto which the appearance of seed mucilage and other morphological characters were drawn and analyzed (Chapter 4).

5) To study the evolutionary history of the character of seed mucilage in the family of the *Cistaceae* we drawn the appearance of seed mucilage along the already constructed phylogenetic tree by Guzmán (2009). To test the hypothesis that mucilage secretion in this family is related to open and stressful habitats we analyze the association of mucilage secretion character to the ability of plants to colonize high competitive habitats under experimental conditions (Chapter 5).

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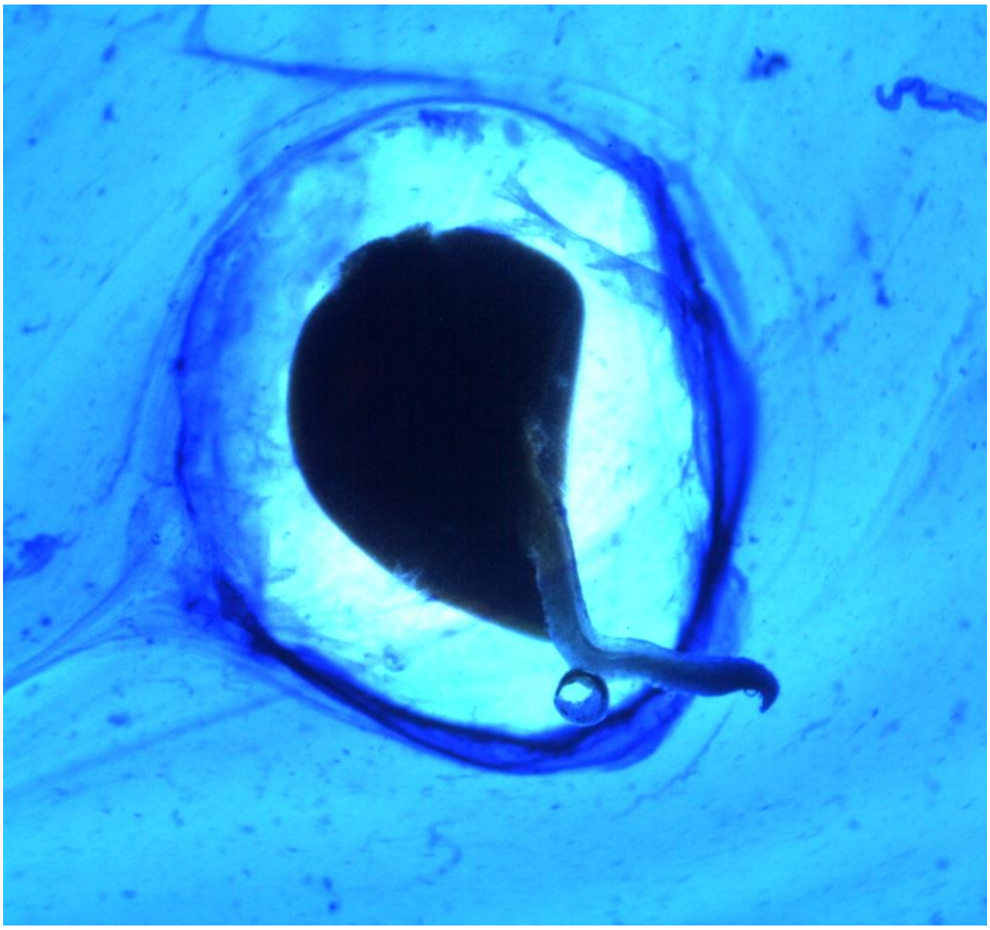
Mucilage secretion, an adaptive mechanism to reduce seed removal by soil erosion?



CHAPTER 1

Abstract

Diaspores of many plant species inhabiting open vegetation in semiarid environments secrete mucilage after wetting (myxospermy) that glues the diaspores to the ground and prevents movement when the mucilage dries. In the present paper we test whether mucilage secretion can be considered a selective response to soil erosion in plant species inhabiting semiarid environments.

We relate the amount and type of mucilage secretion by seeds of *Helianthemum violaceum* and *Fumana ericifolia* (Cistaceae) with the number of raindrop impacts needed to remove these seeds after gluing them with their own mucilage to the ground and the time that these seeds resist water runoff without detaching. We also compare the amount of seed mucilage production by plants growing in habitats without erosion and plants affected by severe erosion by fitting mixed effect models.

Our results show an important phenotypic variation in the amount of mucilage secretion in both species; but suggest that the effect of mucilage secretion in the rate of seed removal by erosion is species and mechanism dependent. For *F. ericifolia*, the amount of mucilage secreted by the seeds is directly proportional to their resistance to raindrop impacts and is positively related to the intensity of the erosive processes that the plants experience. Nevertheless, all the seeds resist the force of runoff during 60 minutes irrespective of the amount of mucilage they produce. In *H. violaceum*, mucilage secretion *per se*, and not the amount of mucilage produced by the seeds has an effect on the rate of seed removal by erosive processes. Furthermore, cellulosic fibrils were found only in the mucilage of *F. ericifolia* but not in *H. violaceum*. Overall, our results only partially support the hypothesis that a selective response to soil erosion exist.

1. Introduction

There is consensus that the cause of natural selection is any environmental or biotic agent that results in differential fitness among phenotypes (see MacColl, 2012), for a recent review). However, current understanding of the causes of natural selection is still poor and it is necessary to deepen our knowledge of selective agents by focussing on the structure and consequences of ecological variation on the traits we suspect are under selection (Siepielski, DiBattista and Carlson, 2009). We can tackle this by discovering how selection changes the phenotypic distribution of traits in nature and why selection operates in this way (Wade and Kalisz, 1990).

The light environment, water conditions, temperature, mineral nutrition, competition, herbivory, plant pathogen interactions, pollinator and dispersal services, and perturbations have been frequently cited as selection agents for plants (Gurevitch, Scheiner and Fox, 2002; Herrera and Pellmyr, 2002; Niklas, 1997; Pausas and Keeley, 2009). However, soil erosion has not yet been considered an agent of selection for plants and is usually only viewed as an environmental problem (Boardman, 2006). Arid and semi-arid regions of the world are characterised by sparse vegetation cover and in these regions most of the annual precipitation is concentrated into a small number of high intensity events, which have a high potential for soil erosion (Rodriguez-Iturbe and Porporato, 2004; Thompson, 2005; Whitford, 2002). Seeds resting on the soil surface of hillslopes after seed dispersal are at risk of losing opportunities for plant recruitment due to erosive processes that move them downhill, where they can become buried (García-Fayos et al., 1995) or suffer strong competition with more competitive seedlings or from pre-established vegetation (Cantón et al., 2004). Under these circumstances, anchoring to the ground surface can help seeds of stress tolerant and less competitive plant species to resist removal by erosive processes and therefore increase their fitness.

There are two main mechanisms by which soil erosion proceeds: soil particle splash detachment by raindrops and detachment and transport of soil particles by runoff for surface wash along hillslopes (Lal, 2001). The severity of these soil erosive processes depends on the characteristics of the rainfall (duration, intensity, amount, drop size), on the physical and chemical properties of the soil (aggregation, infiltration, and hydraulic conductivity), and on the topography of hillslopes (angle and length of the slope, Thornes, 1985). Similarly to what happens with the mineral particles of soil, the susceptibility of seeds to be removed from the ground by erosion mainly depends on seed size (García-Fayos and Cerdà, 1997), but also on characteristics such as shape, presence of appendages ,and the ability to secrete mucilage from the seed coating or the pericarp (myxospermy; García-Fayos, Bochet and Cerdà, 2010; Wang et al., 2013).

Once the myxospermic diaspores reach the soil surface, light rain as well as dew or even ground humidity, are enough to induce mucilage secretion in seconds or minutes (Meike Engelbrecht personal observation; Huang et al., 2008), and after drying ,it anchors the seed to the surface it is resting on (Van Rheede van Oudtshoorn and Van Rooyen, 1999). Two main types of mucilage have been recognized: “true” mucilage, consisting of almost exclusively of pectin, which occurs for instance in the genus *Linum*; and “cellulosic” mucilage, consisting of pectin and cellulose fibrils, which occurs in the genus *Salvia* and *Artemisia*. Pectin increases the amount of water and viscosity around the seed coat and once it dries result in adherence of the seed coat to the surface it rest on. Cellulose fibrils increase the amount and strength of the contact between the seed and the surface, thus enhancing the anchoring power of the pectin (Grubert, 1974; Gutterman, Witztum and Evenari, 1967; Kreitschitz, 2009; Werker, 1997; Witztum, Gutterman and Evenari, 1969).

Mucilage secretion by diaspores was initially related to seed dispersal functions in plants inhabiting semiarid environments (Friedman and Stein, 1980; Zohary, 1962). However, other functions as protecting seeds from predation, flooding, and the regulation of germination have been demonstrated (Gutterman and Shem-Tov, 1996; Gutterman and Shem-Tov, 1997a; Witztum et al.,1969). Several authors then proved that mucilage secretion significantly hinders seeds from predation by granivorous ants (Engelbrecht and García-Fayos, 2012; Fuller and Hay, 1983; Gutterman and Shem-Tov, 1997a) and reduces the probability of seed removal by soil erosion, especially small seeds (Ellner and Shmida, 1981; García-Fayos et al., 2010; García-Fayos and Cerdà, 1997). Other studies found that the proportion of plant species whose diaspores are myxospermic varies among plant communities that differ in the severity of soil erosion and also that this proportion correlates with those soil properties linked to runoff generation (García-Fayos, Engelbrecht and Bochet, 2013). Likewise, other authors showed that mucilage secretion plays a role in the germination of seeds in semiarid environments by increasing the water retention of seeds and increasing the seed-to-soil-particle contact (Gutterman and Shem-Tov, 1997b; Huang et al., 2008; Lu et al., 2010).

Recent reviews have examined the functions proposed for mucilage secretion in diaspores (Kreitschitz, 2009; Western, 2012; Yang et al., 2012) and from them emerge the view that the adaptive value of mucilage may vary between plant lineages and may respond to several not mutually exclusive forces – so hindering the establishment of general patterns. Away to disentangle the role of mucilage secretion of a plant species or a plant lineage is to analyse the variation in the amount of mucilage secreted by diaspores along gradients of selected environmental factors that presumably affect this amount (i.e. gradients of increasing climatic drought crossed with gradients of increasingly severe soil erosion). A complementary or alternative approach would be to analyse the variation in mucilage secretion in plants whose seeds have traits that simultaneously preclude some of the possible functions of mucilage. For example, in the case of plants whose seeds show physical dormancy or deep physiological dormancy (sensu Baskin and Baskin, 1998) and then require long periods of cold or warm stratification before germinating, it is unlikely that the role of mucilage secretion is related to enhance the rate or the speed of germination. Similarly, under sunny conditions in temperate and tropical climates the water captured by the mucilage is lost less than one hour after rainfall due to evaporation (Grubert, 1981; Ryding, Harley and Reynolds, 1992), thus making improbable the use of this water for germination.

To test the hypothesis that myxospermy is an adaptive response of plants to soil erosion it is necessary to prove that: (i) the amount of mucilage produced per seed is positively related to the reduction in the rate or in the distance of seed removal by soil erosion (mechanism selection); and (ii) increasing the intensity of soil erosion in the environment where plants develop produces a positive phenotypic selection at the population level in the amount of mucilage secretion (character selection) with other potential selective forces being equal.

In the present paper, we analyse the relation between mucilage secretion by seeds and the intensity of soil erosion processes in several populations of *Fumana ericifolia* and *Helianthemum violaceum* (Cistaceae) and discuss the results at the light of the type of mucilage secreted by these species. Cistaceae species are characteristic of open plant communities in dry and sunny temperate habitats and their seeds have hard seed coats that impede germination until it is removed or scarified (Baskin and Baskin, 1998; Ferrandis, Herranz and Martínez-Sánchez, 1999; Guzmán and Vargas, 2009; Herranz, Ferrandis and Martínez-Sánchez, 1999; Moreira et al., 2010; Thanos et al., 1992; Thompson, 2005). In the family of the Cistaceae, pectin has been supposed to be the main component of mucilage, but for *Fumana laevipes* cellulose components have been described as well (Grubert, 1981).

2. Material and Methods

2.1 Type of seed mucilage

To detect differences in the type of seed mucilage of *F. ericifolia* and *H. violaceum* we observed wetted seeds of both species under the binocular. Chemical staining was used to distinguish pectin mucilage from cellulose mucilage. We therefore used methylene blue (0.1% solution) to detect the cellulose content, (Kreitschitz, 2009; Kreitschitz and Vallès, 2007) and ruthenium red was used to stain the pectin part of the mucilage red (Hanke and Northcote, 1975; Western et al., 2001; Western, Skinner and Haughn, 2000).

2.2 Seed resistance to drop impact

To measure the resistance to direct drop impacts of mucilaginous seeds adhered to the ground, we applied the water drop test method to fully developed seeds of *Helianthemum violaceum* and *Fumana ericifolia*. This method simulates the rain drop impact that seeds undergo in the field during storms and is usually used to test the stability of soil aggregates to erosion forces (Imeson and Vis, 1984). Seeds of both species were equally collected from the different study areas (see Section 1.4) and then mixed. We measured mucilage production by submerging 83 fully developed seeds of *F. ericifolia* and 104 fully developed seeds of *H. violaceum* in distilled water until the maximum mucilage production of each seed was reached (in less than 45 minutes). Although the technique may not reflect the exact natural conditions that seeds experience, this method gives us an idea about the amount of mucilage produced by each seed and permits us to homogenise the experimental conditions. High resolution photos were taken with an incorporated camera in a binocular (Leica LED 2500 80x) at an augmentation of 1 x 80. The projected area of the seed surface and the mucilaginous layer were measured with the image analysis software SigmaScan Pro Version 5 (1999). Then, seed surface was used as a proxy of seed size and relative mucilage production per seed was then calculated as the quotient of mucilage area by seed area.

After taking the photo, each seed was immediately placed on a wet filter paper (2 mm thick) and completely air-dried. The filter paper with the seed was placed on an inclined plastic surface (20º angle) and the number of drop impacts required to detach each seed from the filter paper was counted. The inclination angle was selected because it is in the range of the slope angles where plant populations of highly eroded habitats develop (see Section 1.4). Drops were produced by a pipette with a water supply system with a constant head and fell on each seed with a frequency of approximately one drop per second from a height of 1 m. Mean drop size diameter (D_{50}) was 5 mm which is in the range of rainfall drops during medium intensity storms with a return period of around five years in eastern Spain (Arnaez et al., 2007; Cerdà, Garrigós and García-Fayos, 2002). The number of direct drop impacts needed to detach the seeds was evaluated separately for each species (*F. ericifolia* and *H. violaceum*) as they varied significantly in seed size and absolute and relative mucilage production (Table 1). Linear and quadratic regression models were used to fit the relation between the relative mucilage production as well as seed size with the number of drop impacts needed to detach the seeds. Statistical analyses were performed with the SPSS statistical package v. 19.0 and residuals were checked for normality.

2.3 Seed resistance to runoff

To simulate runoff, water flux experiments were conducted under laboratory conditions with a 250 cm long and 30 cm wide methacrylate flume modified from Poesenet al*.* (1999).The slope of the flume was set to an inclination of 25º and tap water was used to generate runoff. The strength of the overland flow caused by runoff was estimated by means of the flow shear stress, which was calculated following the equation described in De Baetset al*.* (2007). In the experiments, a flow shear stress of 5Pa was applied because it was the minimum flow strength under which seed removal was observed and, at the same time, it is in the range of values that produces detachment and transport of particles on hillslopes– but is still below the critical flow strength that forms rills and gullies (1.8 to 10.6 Pa in Poesen et al., 2003).The return period of rainfall events with this intensity in western Mediterranean semiarid environments is 10 years, but for some coastal regions this period is shorter (Grove and Rackman, 2001). A thin layer of sand particles between 0.02 and 1 mm, with the major proportion between 0.25 and 0.5 mm, was previously glued to the surface of the flume channel to provide roughness approximating natural conditions.

The quantity of mucilage in seeds was measured to establish a relation between the strength of seed adherence and the resistance to runoff. Fully developed seeds of *H. violaceum* and *F. ericifolia* collected in the study areas (see Section 1.4 for details) were moistened to provoke the secretion of mucilage and then photographed to measure the relative mucilage production and seed size as described in Section 1.2. Ten seeds were then placed on 50 x 30 x 0.5 cm plastic sheets over a thin layer of the same sand material glued to the bottom of the flume. Seeds were arranged along a horizontal line, at 1.5 cm intervals, and at 8 cm from the left and right margin and 10cm from the upper margin of the sheet to prevent edge effects. For each species ten plastic sheets with 10 or 11 seeds each (total of 104 seeds) were used. Once the seeds were dried, and in consequence glued to the sand layer, the sheet was placed in the flume, at 85 cm from the water flux entry, in a cavity equalling the size of the sheet so that the sample surface was at the same level as the flume surface. Once the water flow discharge began, time to detachment from the plastic sheet was measured for each seed with a maximum run time of 60min per experiment. We designed a complementary test with seeds of the two species in which the mucilage layer was eliminated and their resistance to being removed by runoff was compared with that of intact seeds using the same method as the previous experiment. In each experiment we used seeds of similar weight to enable a comparison of the results (García-Fayos et al., 2010). To eliminate the mucilage layer, seeds were first wetted for 15 minutes and then rubbed on filter paper until the mucilage was removed.

Linear and quadratic regression models were used to fit the relation between the relative mucilage production as well as the seed size with the time in minutes needed to detach the seeds. Statistical analyses were performed with SPSS statistical package v. 19.0 and residuals were checked for normality.

2.4 Differences in amount of seed mucilage from plants growing under contrasting erosion regimes

We looked for variations in the amount of mucilage produced by seeds of *F. ericifolia* and *H. violaceum* collected from plants growing in several populations that experienced contrasting soil erosion pressures. We also analysed the variations in seed weight (mg) and seed area (mm^2) of these seeds to control for the indirect effects that erosion might have on the amount of mucilage through changes in seed size.

The study area was located in the Alfambra River basin (province of Teruel, Spain), which is an area of 4000 m^2 that lies at an altitude of 900 to 1100 m a.s.l. The study site has a yearly average temperature of 11.9 ºC and precipitation of 368 mm (see García-Fayos and Bochet, 2009, for a more detailed description of the study area).

We selected two geomorphological conditions, almost flat highlands (<5º slope angle) and their associated hillslopes (25-30º), as surrogates for ‘no erosion’ and ‘severe erosion’ treatments respectively. For ‘no erosion’ sites we used forest clearings larger than 0.05 km^2 and for ‘severe erosion’ sites we used sectors of hillslopes longer than 100 m length. All sites were southerly (see García-Fayos and Bochet, 2009, for more details). In spring and summer 2009 we identified three populations per treatment of the selected species, each at least 500 m from any other sampled slope and all containing more than 100 individuals. In each population we collected mature seeds from15 individuals, each at least 5 m distant from other sampled plants. Under laboratory conditions, we weighed the seeds and measured the size (seed area in mm^2) and the relative mucilage amount (surface of secreted mucilage/seed surface) of 10 fully developed seeds from each individual plant as described in paragraph 1.2.

Because the seeds of the studied species were food items for granivorous ants (see Engelbrecht and García-Fayos, 2012), ants may also potentially exert a selective pressure on the amount of mucilage secreted by the seeds. However, nest density of granivorous ants – as a surrogate of seed harvesting pressure –in the ‘no erosion’ and ‘severe erosion’ hillslopes was 97.6 ± 36.4 and 81.8 ± 15.8 nests/ha respectively (Mean ±SE, see García-Fayos et al.,2013) and did not significantly differ between them (t-test= 0.1512, df = 13.977, p-value = 0.882).

2.4.1 *Statistical analysis*

We fitted linear mixed models to analyse the relative amount of mucilage from the seeds. All these models included an erosion regime where plants grow as a fixed effect (‘no erosion’ vs. ‘severe erosion’) and various alternatives for random effects: i) the individual plant from which the seeds were collected; ii) the population where these plants develop; iii) the individual plant nested within the population; and iv) no random effects. Akaike’s information criterion was used to choose among competing statistical models, as suggested by Zuur (2009) and Crawley (2007). We performed linear mixed-effect models using the R ‘lmer’ function from ‘lme4’ (Bates, Maechler and Bolker, 2011). We used the ‘lmer’ function as it fits a greater range of models and is more reliable than other similar functions (Bates, 2005). We constructed the models using the restricted maximum likelihood (REML) estimation procedure (Zuur et al., 2009). The significance of the fixed effects was evaluated by means of likelihood ratio tests by calling the function ‘anova’ to compare models with and without the factor being tested – which was previously fitted using maximum likelihood (ML) estimates (Crawley, 2007; Zuur et al., 2009). In addition, to assess the uncertainty of parameter estimates from the linear mixed models, we ran 10000 Markov Chain Monte Carlo (MCMC) samples using the function mcmcsamp in lme4, estimating the posterior probability density of model parameters. Parameter estimates for which the 95% confidence interval (95% CI, defined by the 2.5 and 97.5% quantiles from the resampled distributions) did not overlap with zero were considered significant. The confidence interval was calculated using the HPDinterval function in 'lme4'.

Differences in seed weight and size under contrasting soil erosion pressures were evaluated for the two species with mixed effect models following the same steps as described above. All statistical analyses were carried out in R version 2.12.2 (R Development Core Team, 2011).

3. Results

3.1 Type of seed mucilage

We found differences in the type of seed mucilage. Ruthenium red stained the mucilage of *F. ericifolia* and *H. violaceum* seeds homogeneous pink, revealing the pectin matrix of the mucilage in both species (Figure 1 A-D). After staining the seeds with methylene blue, dark blue cellulose strands were identified in the mucilage of *F. ericifolia* (Fig. 2 A-B) but not in the seeds *of H. violaceum* (Figure 2 C-D). Seeds of *H. violaceum* seeds stained slightly blue in the outermost surface of the mucilage but no cellulose fibrils were coloured. The mucilage of *F. ericifolia* seeds therefore present a heterogeneous system made of pectin matrix with cellulose strands.

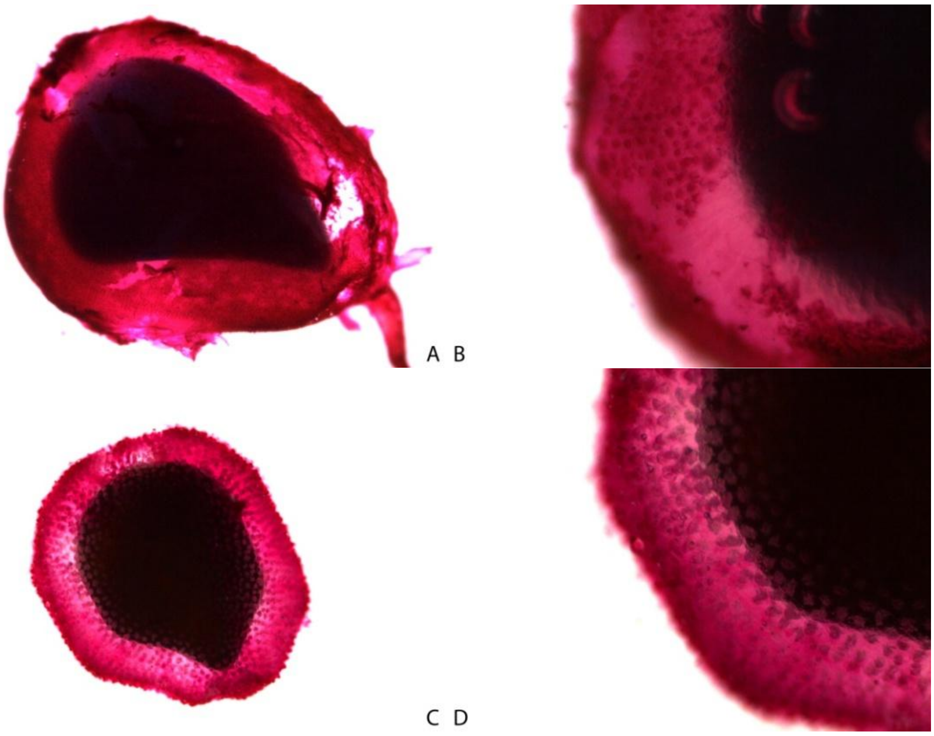


Figure 1: Structure of the mucilage stained with ruthenium red. Intense pink stained polysaccharides are visible in the mucilage layer around the seed.

A) *F. ericifolia* seed 1.25 x 80 amplification, B) *F. ericifolia* seed 6.3 x 80 amplification, C) *H. violaceum* seed 1.5 x 80 amplification, D) *H. violaceum* seed 6.3 x 80 amplification.

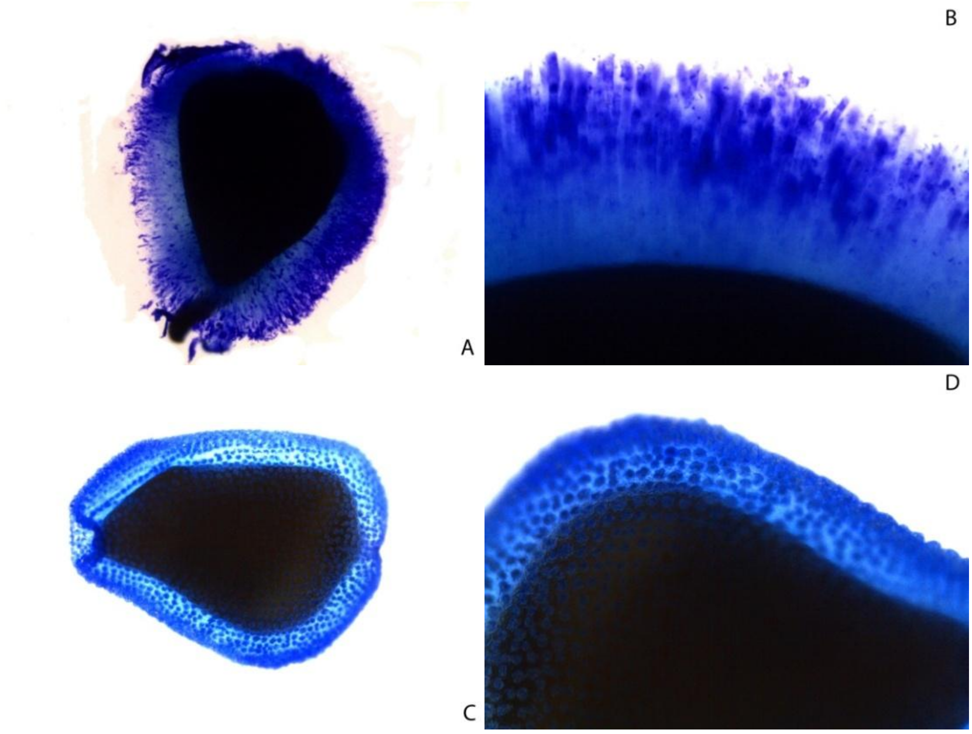


Figure 2: Mucilage formation and staining results with methylene blue. Outermost surface is stained blue and cellulose fibrils are stained dark blue. A) *F. ericifolia* seed with cellulosic fibrils clearly stained dark blue (1.25 x 80 amplification), B) *F. ericifolia* seed mucilage at 8 x 80 amplification. Thick helicoidal strands of

cellulosic fibrils, partially uncoiled, are stained dark blue, C) *H. violaceum* seed mucilage without cellulose fibrils, only the outer layer of the mucilage is stained blue (1.5 x 80 amplification), D) *H. violaceum* without cellulosic mucilage, only the surface is stained blue (6.3 x 80 amplification).

3.2 Seed resistance to drop impact

The number of water drop impacts needed to detach the *F. ericifolia* seeds are directly related to their relative mucilage production. After applying different regression models, the power relation was the best fit between the relative mucilage production of a seed and the number of drop impacts needed to detach it (R^2= 0.482; F1;82 = 75.49; p< 0.0001; Fig. 3a) and thereby indicating an exponential effect of mucilage secretion on the seed resistance to drop impacts. For *H. violaceum,* this relation follows a bell shape distribution (Fig.3b) indicating a lack of relation between seed mucilage secretion and the susceptibility of a seed to be moved by drop impacts.

The number of water drop impacts needed to detach the seeds of both species had not related to the variation in seed size. Both species showed a cloud of points of seed size and numbers of drop impacts needed to detach to which no regression could be fitted (data not shown).

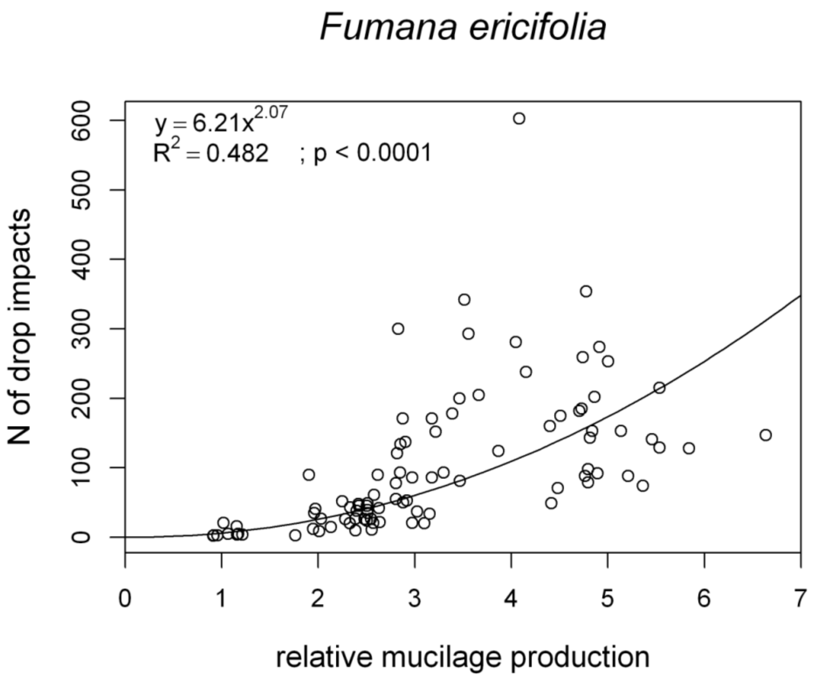


Figure 3a: Resistance of adhered seeds of *Fumana ericifolia* to detachment by drop impact (0.05g drops of water from a height of 1m). Seeds had been glued to filter paper with their own previously secreted mucilage and air dried. Relative mucilage was calculated as the total mucilage (in mm^2) divided by the seed area (in mm^2). Each point reflects one separately tested seed (N=83).

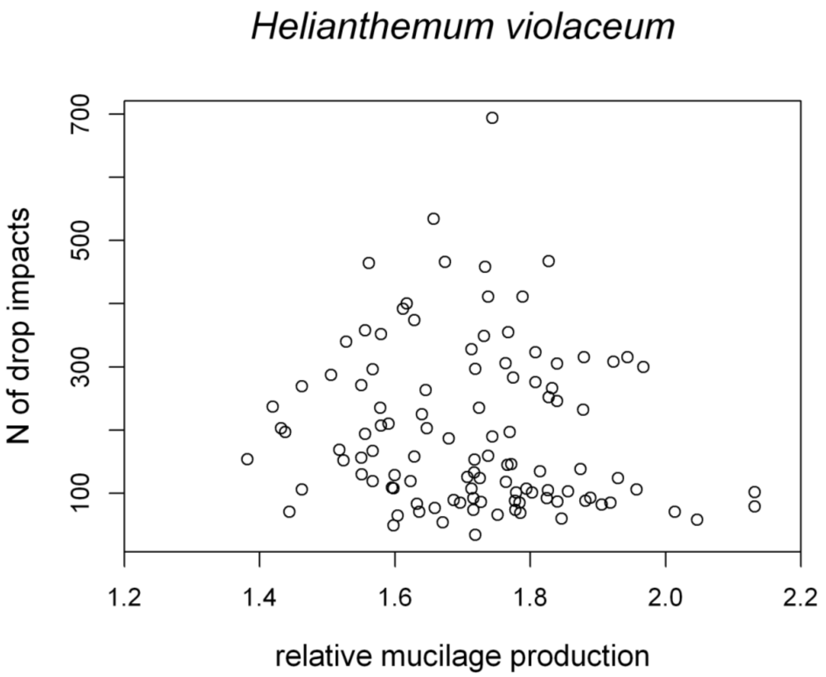


Figure 3b: Resistance of adhered seeds of *Helianthemum violaceum* to detachment by drop impact (0.05g drops of water from a height of 1m). Seeds had been glued to filter paper with their own previously secreted mucilage and air dried. Relative mucilage was calculated as the total mucilage (in mm^2) divided by the seed area (in mm^2). Each point reflects one separately tested seed (N= 104).

3.3 Seed resistance to runoff

All the seeds of *F. ericifolia* remained glued to the ground until the end of the water flux experiments (60 min) irrespective of the amount of mucilage the seeds produced; and 66.4% of the *H. violaceum* seeds remained glued until the end of the experiment. However, we found no relation between the times that these seeds resisted runoff and their relative mucilage production (Fig. 4). Also when relating the seed size of both species with the time that seeds resisted to runoff, we did not find any relation (data not shown). When we performed the experiment with demucilaged seeds of *H. violaceum*, most of the seeds were removed from the ground by runoff in just a few minutes and again we did not find a relationship with the amount of mucilage, indicating that mucilage secretion per se helps seeds of this species resist removal by runoff – but that there is no direct relationship with the amount of mucilage secretion. We could not perform the experiment with demucilaged seeds of *F. ericifolia* as the mucilage layer of the seeds cannot be eliminated without strongly damaging the seed coat, thus influencing the condition of the seed to resist runoff.

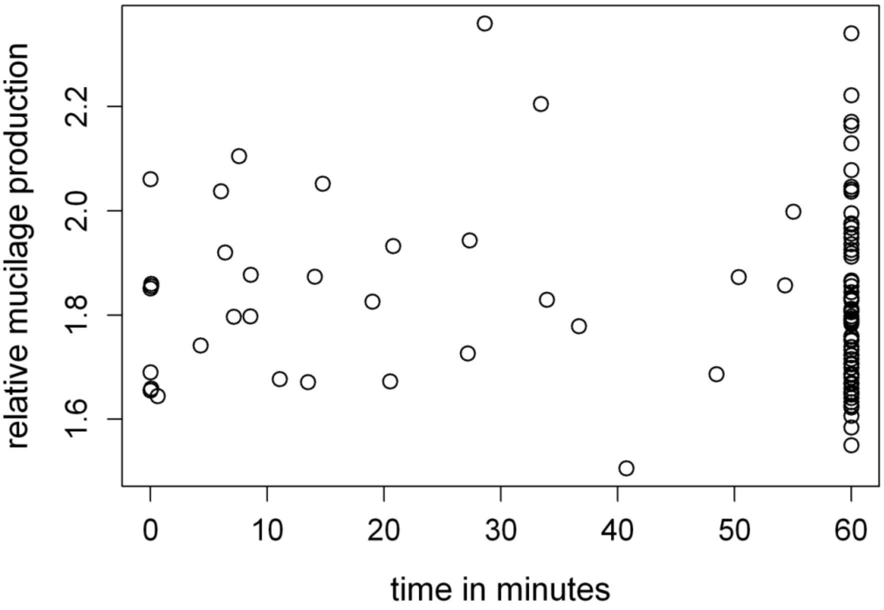


Figure 4: Resistance of adhered seeds of *H. violaceum* to detachment by runoff in a hydraulic flume with an inclination of 25º and flow shear stress of 5 Pa for a maximum of 60 minutes. Seeds were glued to the bottom of the flume channel with their own previously secreted mucilage and air dried. Relative mucilage was calculated as the total mucilage (in mm^2) divided by the seed area (in mm^2). Each point reflects one separately tested seed (N= 104).

3.4 Differences in amount of seed mucilage from plants growing under contrasting erosion regimes

The relative mucilage production of *F. ericifolia* seeds was lower (3.329 ± 0.031) for seeds collected from ‘no erosion’ plant populations than for seeds from ‘severe erosion’ plant populations (3.697 ± 0.033, 95% CI: –0.5805, –0.1875). Seeds of *H. violaceum* from plants in ‘no erosion’ and ‘severe erosion’ populations produced very similar amounts of mucilage (1.744 ± 0.006 vs. 1.727 ± 0.006, 95% CI: –0.0113, 0.0469).

When we evaluated the mixed models using the amount of mucilage secreted by each seed as the response variable, the model with the lowest AIC for *F. ericifolia* was that which used the individual plant from which the seed was collected as a random term (AIC= 903.74). However, for models using the individual plant nested within a population, those with the population as random terms, and the model using no random term, obtained higher AIC values (905.74, 1104.17 and 1151.36 respectively). For the *H. violaceum* species*,* the best adjusted model was also the one in which individual plants from which the seeds were collected were used as a random effect (AIC=-1038.47). The models with the individual plant nested within the AIC population (-1036.47) and those using only population as random terms, or no random terms, gave poorer fitted models (AIC= -924.59, AIC =-920.99, respectively). For both species, the ANOVA analysis indicated that the model with the lowest AIC was significantly better than the next lowest AIC model (p< 0.0005 in all cases).

Our results also show that the effect of the treatment (fixed term) was significant only for *F. ericifolia* and not for *H. violaceum* (likelihood ratio test p= 0.0109, p= 0.3149, respectively), indicating a significantly higher mucilage amount in seeds collected from plants living in the severe erosion site for *F. ericifolia* (95% CI: –0.5805, –0.1875) but not for *H. violaceum* (95% CI: –0.0113, 0.0469).

The selected terms in the mixed models were the same when we used seed weight and seed area as response variables. For both species, the lowest AIC was found when the plant individual from which the seeds were collected was used as random effect; while the models with the individual plant nested within the population, models using only population as random terms, and models with no random term gave poorer fitted models (Supplementary Table 1). We found significantly heavier seeds in the 'severe erosive' treatment than in the 'no erosion' treatment for both species (*H. violaceum*: 0.86 ± 0.0083 mg vs. 0.72 ± 0.0069 mg and *F. ericifolia:* 2.22 ± 0.049 mg vs. 1.74 ± 0.081mg respectively). However, we found significant differences in seed area between the two erosive sites in *H. violaceum* (95% CI-0.206; -0.123; 1.33 mm^2; 1.16 mm^2, respectively), but not for *F. ericifolia* (95% C -0.152; 0.137).

Table 1a: Overview of the range (min-max) of seed mass, seed area, mucilage area, relative mucilage area, minimum time to secrete mucilage and time till maximum mucilage is released from seeds of *Fumana ericifolia* and *Helianthemum violaceum* for the **flat geomorphologic site with no erosion**. Seeds measured (N) were recollected in the different study sites from year 2007 to 2009.

Species	dry seed weight (mg)	seed area (mm^2)	mucilage area (mm^2)	relative mucilage area (mucilage area/ seed area)	Minimum time to mucilage secretion (min)	Time till maximum mucilage is secreted (min)	N
F. ericifolia	0.57 – 2.58	1.41 – 4.18	2.24 – 12.93	1.22 – 5.11	1.0	5	480
H. violaceum	0. 40 –1.06	0.69 – 1.69	1.31 – 2.84	1.40 – 2.23	1.5	45	451

Table 1b: Overview of the range (min-max) of seed mass, seed area, mucilage area, relative mucilage area, minimum time to secrete mucilage and time till maximum mucilage is released from seeds of *Fumana ericifolia* and *Helianthemum violaceum* the associated hillslopes with **severe erosion**. Seeds measured (N) were recollected in the different study sites from year 2007 to 2009.

Species	dry seed weight (mg)	seed area (mm^2)	mucilage area (mm^2)	relative mucilage area (mucilage area/ seed area)	Minimum time to mucilage secretion (min)	Time till maximum mucilage is secreted (min)	N
F. ericifolia	0.90 – 3.17	1.73 – 4.44	6.08 – 15.8	2.03 – 6.80	1.0	5	464
H. violaceum	0. 53 –1.35	0.69 – 1.97	1.38 – 3.27	1.42 – 2.45	1.5	45	461

4. Discussion

The results of our study incompletely support the hypothesis that myxospermy can be considered as an adaption to soil erosion. Results clearly indicate that myxospermy reduce the probability of seeds of both species being removed by mechanisms of soil erosion (drop impact and runoff). However, the quantifiable effect of mucilage seems to be mechanism and species dependent.

So, in *F. ericifolia* the relative amount of mucilage produced per seed is positively and strongly related to the reduction of seed removal by rain drop impact (mechanism selection); and, at the same time, the phenotypic variation of mucilage secretion was related to the intensity of soil erosion experienced by mother plants (character selection). However, no relation was found between the amount of mucilage released by the seeds and the time they resisted runoff.

For *H. violaceum*, despite the close phylogenetic relation between both species (Guzmán and Vargas, 2009), results did not support any of the assumptions of mechanism and character selection that we found in *F. ericifolia*. Although we found a clear difference in *H. violaceum* between intact and demucilaged seeds in relation to removal rate by drop impact and runoff, we did not find any relation between the amount of mucilage secretion of intact seeds and the removal rate by these erosive mechanisms. Consistently, the erosive environment experienced by mother plants had no influence on the amount of mucilage secreted by the seeds.

Differences in the kind of mucilage substances could be in the basis of the variation in adherence ability between both species, as stated by Grubert (1974), Werker (1997) and Kreitschitz (2009). The mucilage of *F. ericifolia* seeds has cellulosic fibrils embedded in the pectin layer while the mucilage of *H. violaceum* seeds is composed only of a pectin layer. Cellulosic strands in the matrix of the pectin mucilage are supposed to supply better adherence of the pectins to the seed and aids a stronger adherence of the seed to the soil surface than simple pectic mucilage (Grubert, 1974; Gutterman et al., 1967; Witztum et al., 1969). The seeds of *F. ericifolia* stayed attached to the soil surface under heavy runoff in a higher rate than that of *H. violaceum* and also the mucilage of *F. ericifolia* seeds cannot be removed from the seed coat but it is easily removed from *H. violaceum* seeds. We propose then that the mucilage composed only of pectin, such as of *H. violaceum*, has not been moulded by the forces of erosion, but yet has sufficient adhesion power to resist erosion events of average intensity. Therefore, we consider that other factors not tested in our study could influence their phenotypic variation.

Results also show that mother plants of both species exert considerable control over seed size and the amount of mucilage secretion, as indicated by the fitted mixed models. Seed related traits have often been found to be more strongly controlled at an individual than at a population level (as, for example, with seed output, size and dormancy). Adaptive and non-adaptive explanations have been proposed to explain this pattern. Intraspecific variability of reproductive traits can help species recruit under variable temporal and spatial environmental conditions, as may be the case for patchy vegetation in semiarid environments (Aguiar and Sala, 1999; Alados et al., 2010, Baraza, Arroyo and Garcia, 2010; Halpern, 2005; Moreira and Pausas, 2012). Under the erosive conditions that prevail in these environments, a quantitative increment in relative mucilage production may benefit the seeds only if longer or stronger adherence translates to a greater chance of the seeds germinating and establishing themselves in the environmental conditions of the sites where they were primarily dispersed. We did not obtain direct empiric or experimental evidence in the studied populations, but the spatial pattern of seedling recruitment in the *Fumana* species strongly suggests that it is the case. Arnan et al. (2010) found that 88% of all new individuals that established themselves during three years did so at distances of less than 0.5 m from the adult individuals. In the closely related phylogenetic species *F. thymifolia*, Jump et al. (2009) found a very limited seed dispersal within the population after studying the within-population genetic structure using amplified fragment length polymorphism (AFLP). We cannot discard that the individual variation in the amount of mucilage secretion we found may also be the result of differences in resource acquisition among plants as a direct consequence of the spatial heterogeneity of soil-related resources in these environments, which can translate directly or indirectly to the mucilage secretion ability (through seed size).

To consider erosion an agent of selection, plants under selection need to experience enough erosion events causing different seed survivals. Since rainfall with drop sizes as used in the experiments have an approximately five-year recurrence (see Section 2.1) and *Fumana* species have a mean life span of 15-20 years (Kovács, Kovács-Láng and Babos, 2002), then *Fumana* plants should experience 3-4 events of that magnitude during their lives. Superficial water flow in contrast, with intensities such as that used in the experiments, usually occurs only once in the life of the studied plants – and so selection pressure would be weaker than in the case of raindrop impact. Still, in the experiments of runoff we detected that the adhesive power of dried mucilage was strong enough to make seeds resistant to high intensity runoff events, as all *F. ericifolia* seeds, and 66.4% of *H.violaceum* seeds stayed glued until the end of the experiment.

Selective pressure towards stronger adherence might only be working in very extreme events– but those events have return periods that exceed the life of these plants. Moreover, during these extreme events the entire soil maybe eroded due to the formation of rills and gullies (Poesen, 2003) meaning that seeds would be removed together with the eroded soil layer– independently of the relative amount of mucilage produced.

In conclusion, our results show that mucilage secretion reduces seed removal caused by erosive mechanisms that operate in natural conditions such as rain drop detachment and removal by water runoff. But our results also indicate that the adaptive value of mucilage secretion seems to depend on the species and the erosion mechanisms involved. Only in one of the two species we studied (*F. ericifolia*) we find that the reduction in seed removal due to raindrop impact is proportional to the amount of mucilage secreted – and also that the greater the strength of the erosion processes that plants experience, the more mucilage was produced by their seeds. However, the close relative species *H. violaceum* did not fit any of the assumptions of our hypothesis and it is possible that in this species mucilage secretion had been shaped by selective pressures not related to erosion.

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Supplementary Table 1: Effects of erosive pressure on seed mucilage secretion, seed weight, and seed size derived from generalised linear mixed-effects model with individual plant and populations from which seeds were collected fitted as random effects for both studied plant species.

Supplementary Table 1a: *F. ericifolia*

Relative mucilage	Random effects	AIC	p-value
	Individual	903.74	<0.0005
	Population	1104.17	1
	Individual in population	905.74	1
Seed weight			
	Individual	-1819.7	<0.0005
	Population	-1730.0	1
	Individual in population	-1817.7	1
Seed size	Individual	523.24	<0.0005
	Population	756.71	1
	Individual in population	525.24	1

Fixed effect Erosive pressure	95% Credible interval	
	Lower	Upper
Relative mucilage	–0.580	–0.187
Seed weight (mg)	-0.793	-0.212
Seed size (mm^2)	-0.154	0.129

Supplementary Table 1a*: H. violaceum*

Relative mucilage	Random effects	AIC	p-value
	Individual	-1038.47	p< 0.0005
	Population	-924.59	1
	Individual in population	-1036.47	1
Seed weight			
	Individual	-8499.9	p< 0.0005
	Population	-8347.8	1
	Individual in population	-8497.9	1
Seed size	Individual	-683.15	p< 0.0005
	Population	-443.34	1
	Individual in population	-681.15	1

Fixed effect Erosive pressure	95% Credible interval	
	Lower	Upper
Relative mucilage	–0.0113	0.0469
Seed weight (mg)	-0.178	-0.105
Seed size (mm^2)	-0.208	-0.124

Post-dispersal seed anchorage to soil in semiarid plant communities, a test of the hypothesis of Ellner and Shmida

CHAPTER 2

Abstract

In this paper we test the Ellner-Shmida's hypothesis that in semiarid environments aridity may select for the lack of seed dispersal mechanisms (atelechory) of many plants, whereas post-dispersal selective forces such as soil erosion, seed predation or limitations to water uptake by seeds may select for structures facilitating seed anchorage to the ground (antitelechory).

We analyzed the proportions of species with seed anchorage mechanisms and that of atelechoric species in shrubland colonizing flat areas and hillslopes in two sites differing in climate dryness. Their relation with several soil properties involved in runoff generation, seed-soil contact and water uptake by seeds and with nest density of granivorous ants was also explored.

Our results support the hypothesis that in semiarid shrubland the proportion of species with seed anchorage mechanisms increases because of soil erosion but not because of climate dryness. This is the first time that a direct relation between the proportion of species with seed anchorage mechanisms and soil erosion is shown in plant communities; supporting the view that soil erosion shapes species composition in communities.

1. Introduction

It has been repeatedly observed that long-distance seed dispersal syndromes are less frequent in arid and semiarid than in mesic habitats and even that mechanisms constraining spatial seed dispersal are a common trait of arid and semiarid floras (Van der Pijl 1972; Ellner and Shmida 1981; Van Rheede van Oudtshoorn and Van Rooyen 1999).

Early predictions claimed that plants inhabiting spatially heterogeneous environments benefits from long-distance seed dispersal because it allows plants to reach distant favourable patches and spread extinction risk in space (Levin et al. 1984; Cohen and Levin 1987). Contrary to this view, Stebbins (1971) and Friedman and Stein (1980) argued that the continuous occupation of the few favourable sites that exist for plant development in those environments may be a superior adaptative strategy for plants (mother-site theory). Accordingly, these authors suggested that, in plant communities inhabiting open environments, long-distance seed dispersal syndromes (telechory) should be selected against whereas short-distance seed dispersal syndromes, including both the lack of syndromes for seed dispersal (atelechory) and the presence of syndromes to avoid seeds to disperse (antitelechory), should be favoured. On the grounds of these arguments, spatial restrictions to seed dispersal have been widely proposed to explain the origin and maintenance of patches in semiarid vegetation (Kefi et al. 2008; Pueyo et al. 2008).

There are two groups of antitelechoric mechanisms used by plants to avoid seed dispersal. One of them is based on the control of the time that seeds disperse and the other is based on mechanisms that anchor the seeds to the ground. Ellner and Shmida (1981) suggested that seed anchoring mechanisms should not be considered adaptations to claim the mother site in the same way that atelechory does, as the mother site theory proposed, because in plants with seed anchorage mechanisms seeds need to be dispersed before seed anchoring mechanisms can operate. They argue that “the openness of desert vegetation and the patterns of climatic variation favour atelechory while antitelechory is generally a side-effect of mechanisms whose adaptative value is not directly related to seed dispersal”. They maintain that anchoring mechanisms are adaptative, given that providing fruits or seeds with mechanisms that actively restrict post-dispersal movement has an additional cost to plants in terms of carbon allocation during their development, but they also argue that these mechanisms may be related to post-dispersal events, such as the loss of opportunities for seeds to germinate derived from seed removal by erosion, seed predation or insufficient seed-soil contact in coarse textured soils.

There are only two reputed mechanisms related to seed anchorage on the ground surface in plants living in semiarid areas, trypanocarpy and myxospermy (Van Rheede van Oudtshoorn and Van Rooyen 1999). Trypanocarpic diaspores have the ability to sink into the cracks or holes of the soil surface by means of hygroscopic movements of appendages attached to the upper part of the diaspore. This mechanism keeps diaspores safe from seed harvesters and removal by wind or water erosion, and also increases the chance for seed germination by increasing the contact surface of seeds with soil particles in coarse textured soils (Stamp 1989; Schöning et al. 2004). Typical examples of trypanocarpic species can be found in the Geraniaceae and Graminae families. Myxospermy is the ability of seeds or fruits to secrete mucilage from their testa or pericarp respectively, once they become moistened. It is a common feature in many families of Angiosperms (Grubert 1974; Western 2012). Rain, fog, dew or even soil moisture when soil is at field capacity are effective enough to stimulate the secretion of mucilage (García-Fayos and Cerdà 1997; Huang et al. 2004). The increase of the diaspore surface due to mucilage secretion reduces the probability of the diaspore being removed (García-Fayos et al. 2010) because when the mucilage turns dry, it strongly anchors the diaspore to the surface it is in contact with. Further research proved that mucilage secretion highly reduces seed predation by granivorous ants (Fuller and Hay 1983; Gutterman and Shem-Tov 1997; Engelbrecht and García-Fayos 2012), significantly reduces seed losses by runoff (García-Fayos and Cerdà 1997; García-Fayos et al. 2010) and, similar to trypanocarpy, increases the amount of surface contact between the seed coat and soil, aiding seed hydration and thus facilitating germination (Harper and Benton 1966; Gutterman and Shem-Tov 1996; Chambers et al. 1991).

Semiarid vegetation is characterized by a two-phase structure of vegetated patches and bare soil (Aguiar and Sala 1999). Rainfall in arid and semiarid regions of the world is mostly concentrated into few high intensity events (Rodríguez-Iturbe and Porporato 2004) with the potential to trigger strong soil erosion processes (Poesen and Hooke 1997). Several authors have shown that the interaction of plant growth with soil erosion can explain the origin and maintenance of patchy vegetation in hillslopes (Aguiar and Sala 1999; Puidefàbregas 2005). In these hillslopes, seeds reaching the ground are at risk of being removed downslope by runoff water and deposited in the lower parts of slopes where they can get deeply buried (García-Fayos et al. 1995; Han et al. 2011) or where the seedlings they produce may be affected by stronger competition than those seedlings emerging on the upper and medium parts of the hillslopes (Cantón et al. 2004; Alados et al. 2006). Although there has been less research on the subject, wind erosion also has the potential of putting seeds at risk in the same way as surface runoff (Whitford 2002). Erosion also affects important soil properties related to plant establishment and development. Raindrop splashes break soil aggregates and sheet flow removes the finest particles and organic debris from the upper layer of soil, thus reducing soil fertility, the rate of water infiltration into the soil and the amount of available water for plants (Calvo-Cases et al. 2003; Monger and Bestelmeyer 2006). In addition, granivorous animals, mainly harvester ants, have a strong impact on seed survival in arid and semiarid ecosystems (Louda 1995; Hulme and Benkman 2002).

Consequently, the loss of seed germination opportunities in semiarid slopes because seed removal by erosion, predation or soil degradation can be a major difficulty for plants to overcome and, therefore, post-dispersal mechanisms allowing diaspore anchorage to the ground, burying the seed into the soil or increasing the amount of contact of the surface of seeds with soil particles could be favourable adaptations. At the same time, seed anchorage mechanisms should be the instrument because a new patch of vegetation originate and maintain in hillslopes. Once a plant develops from the anchored seed it can restrain locally the downslope movement of water and sediments, increasing soil moisture and fertility at the microsite where plant established (Cerdà 1997, Puigdefàbregas 2005) and favouring the growth of the plant and the patch, which in turn increases the ability of the patch to restrict the downslope movement of water and sediments.

If, as Ellner and Shmida (1981) proposed, seed anchorage mechanisms, such as myxospermy and trypanocarpy, are the outcome of agents of selection that operate after seed dispersal, but not the outcome of selective forces shaping seed dispersal, then it should be predicted that an increase in the intensity of all, or some, of the agents of selection that operate on seeds once they have dispersed (i.e. soil erosion, seed predation) must lead to an increase in the proportion of plants with seed anchorage mechanisms, but should not affect the proportion of plant species with atelechory seed dispersal syndromes. On the contrary, increasing the intensity of selective forces that shape seed dispersal only (i.e. climate aridity), must lead to an increase in the proportion of species with atelechory syndromes, but should not affect the proportion of plant species with seed anchorage mechanisms.

To test this hypothesis we compared the proportion of plant species with atelechory seed dispersal syndromes and the proportion of species with seed anchorage mechanisms (myxospermy plus trypanocarpy) in plant communities colonizing flat areas and steep hillslopes at both extremes of an aridity gradient that has been proved to negatively affect soil properties (García-Fayos and Bochet 2009). The area comprising the aridity gradient is mainly composed of calcareous clays and marls and characterized by superficial soil erosion processes more influential in shaping the hillslope landscape than those occurring in other materials such as limestone or sandstone (Cerdà 2002). We also explored the relationships between these proportions of species and physical soil properties related to runoff generation (bulk density, as a surrogate of the rate of water infiltration in soils) and to water uptake by seeds (fine sand content, indicative of the amount of contact between the seed surface and the ground, and soil water availability as a surrogate of the amount of available water for seeds to germinate and establish). At the same time, since seed harvesting pressure by ants may also influence our results, we analyzed the current nest density of granivorous ants in the study area as a surrogate of seed harvesting pressure. All other factors being similar, the density of granivorous ants in semiarid areas highly depends on vegetation structure, food availability and microsite conditions for winter survival (Díaz 1991; Arnán et al. 2007) and we have not reason to expect that density of granivorous ants is affected by climate and erosion in the same way that these factors affect plant species composition. We predict that (i) an increase in climate dryness, but not an increase in soil erosion, should increase the proportion of species with atelechory syndromes; (ii) an increase in soil erosion, but not an increase in climate aridity, should increase the proportion of plant species with seed anchorage mechanisms in plant communities; and (iii) the effect of soil erosion on physical soil properties related to runoff generation and water uptake by seeds may explain the effect that soil erosion has on the proportion of plant species with seed anchorage mechanisms.

2. Material and Methods

2.1 Study Area

The study area is located in the Alfambra river basin (province of Teruel, Spain). The basin is 4000 m^2 in area and lies at an altitude of 900-1100 ma.s.l. It is composed mainly of calcareous marls. While the seasonal pattern is homogeneous in the whole basin, the amount of precipitation and average temperature differ between the southern and northern extremes of the basin with marked consequences on the plant communities. The extreme northern area, hereafter “cool&wet” area, has annual average temperature and total precipitation of 10.3 ºC and 484 mm, respectively, and the southern area, hereafter ”warm&dry” area, 11.9 ºC and 368 mm, respectively (data from the 1990-2009 period, provided by AEMET: www.aemet.es).

In spring 2006, we selected and marked one sampling plot (1x20 m) perpendicular to the main slope direction in each of 30 hillslopes in the “cool&wet” and in the “warm&dry” areas, all of them south-oriented. In each area, 15 plots were placed in flat sites (hereafter “no erosion” sites) and 15 in 25-30º steep hillslopes (hereafter “severe erosion” sites). Marked differences in erosion severity exist between flat and steep sites, varying from 0 to 25% rill development respectively (García-Fayos and Bochet 2009); the latter rill density indicates a notorious sign of erosion processes on steep sites (Cerdan et al. 2010). Wind erosion has not been taken into account in our experimental design, because it has been reported as unimportant in the eastern part of the Iberian Peninsula (Solé-Benet 2006). Vegetation cover in all the plots was low (< 50%) and it was mainly composed of dwarf shrubs and some herbs.

Plant species present in each plot were identified and, when necessary, the plots were surveyed several times during the growing season to ensure that all plant species were recorded. From 2006 to 2009 we collected seeds from all species and 10 mature and fully developed seeds per species were checked and assigned to the following categories: trypanocarpy, myxospermy and atelechory. Trypanocarpy corresponds to diaspores with hygroscopic awns or hairs that are able to enter into the soil; myxospermy corresponds to diaspores that secrete mucilage from their testa or pericarp when wet; and atelechory corresponds to diaspores with mechanisms for short-distance seed dispersal (ballistic, barochory, etc.) or diaspores lacking such mechanisms but also lacking mechanisms for long-distance seed dispersal. Since trypanospermy and myxospermy act after seed dispersal, a species can be classified at the same time into the atelechory category and one of the two seed anchoring categories.

At the end of the summer of 2006, we took five soil subsamples 5 cm deep and 300 cm^3 volume at regular distances in each plot, that were mixed up in a single sample per plot. Samples were air-dried and sieved through a 2 mm mesh and fine sand content (mineral fraction size between 0.10 and 0.25 mm) and the soil moisture content available for plants were determined following the methodology proposed by Klute (1986). Additionally, we took three 57.7 cm^3 soil cores per plot for bulk density determination at the same depth as previous soil sampling and the average value per plot was used for comparisons. These soil properties were significantly and negatively affected by both climate dryness and soil erosion in our study area (García-Fayos and Bochet 2009). Bulk density is a key property influencing water infiltration into the soil and it was chosen as a surrogate of runoff generation. The higher the soil bulk density, the lower the soil water infiltration and consequently, in steep hillslopes lying on calcareous marls, the faster and higher the runoff generation (Cerdà 2002). Fine sand content and water availability were chosen respectively as surrogates of the extent of seed-soil contact and the amount of water stored in soils. The relation of these soil properties with the energy that seeds need to obtain water from soils was considered to be direct for fine sand content and inverse for water availability (Williams and Shaykewich 1971). So, the higher the fine sand content and the lower the water availability in soils, the higher the pressure that seeds need to exert in order to obtain enough water for germination and establishment (García-Fayos et al. 2000; Bochet et al. 2007).

To analyze the current nest density of granivorous ants we sampled six hillslopes per treatment and counted all the ant nests of granivorous species in an area of 250 m^2. Ant nests were attributed to granivorous ants after identification of the worker ants or inspecting the vast pile when direct ant identification was not possible.

Analyses of the influence of climate dryness and soil erosion treatments on the proportion of species with seed anchorage mechanisms and the proportion of atelechoric species per plot were performed with Linear Models, considering climate dryness and erosion as main factors and the proportions of atelechoric species and of species with seed anchorage, as response variables. Analyses were firstly performed on the total set of species with seed anchorage mechanisms, either myxospermic or trypanocarpic, and secondly, separately on each of both categories. We checked residuals for departure from normal distribution.

Because the size of the plots we used to sample ant nest density was greater than that used to sample plant species composition, it was not possible to directly analyze the effect of nest density on the proportion of plant species with seed anchorage mechanisms. Instead, we analyzed the effect of climate dryness and seed erosion on ant nest density per hillslope, and compared this result with that obtained from the analysis of the effect of the same factors on the proportion of species with seed anchorage mechanisms per plot. Statistical analyses were performed with the *lm* function of the R package v. 2.14.1 (R Development Core Team at http://cran.r-project.org/ last accessed 25/05/2012).

3. Results

We found a total of 167 plant species in the study area. Of them, 139 were unambiguously assigned to the different dispersal and seed anchoring diaspore categories. Among these, 108 (77%) were considered to be atelechoric and 31 (22%) having any seed anchorage mechanism (26 myxospermic species -several Brassicaceae, Cistaceae, Lamiaceae, Asteraceae, Linaceae, Rosaceae, Plantaginaceae and Aristolochiaceae species- and only 5 trypanocarpic species -four Graminae, *Stipa parviflora*, *S. celakowsky*, *S. lagascae*, *Avenula pratensis* and one Geraniaceae, *Erodium cicutarium*). At the plot level, 67 to 94% of the species have atelechoric diaspores (79% in average, see Table 1) and 17 to 45% of the species have diaspores with anchorage mechanism (30% in average) and the proportion of myxospermic species is higher (15-44%, 26% in average) than that of trypanocarpic species (0-8%, 2.4% in average).

Table 1 shows the variation among plots in the proportion of atelechory and seeds with anchorage mechanisms. The linear model significantly explained the variation in the proportion of atelechoric species (Adjusted $R^2 = 0.1366$; $F_{3, 56} = 4.112$; $p = 0.0105$) indicating that it is affected, although marginally ($p = 0.0753$), by climate dryness (81.2 ± 4.5% (Mean ± SD) in the “warm&dry” plots vs. 77.5 ± 3.9% in the “cool&wet” plots), but that neither soil erosion ($p = 0.3572$) nor the interaction of both factors ($p = 0.4178$) affected the proportion of atelechoric species.

Relative to the proportion of species with seed anchorage mechanisms, the linear model significantly explained a moderate proportion of the variance (Adjusted $R^2 = 0.3481$; $F_{3, 56} = 11.5$; $p < 0.0001$) showing that the proportion of species whose diaspores have anchorage mechanisms is positively affected by soil erosion ($p = 0.0002$) but neither by climate dryness ($p = 0.6616$) nor by their interaction ($p = 0.7426$). On average, the proportion of species with seed anchorage mechanisms is 26.7 ± 4.3% in the “no erosion” plots and 33.5 ± 4.6% in the “severe erosion” ones.

When the proportion of species with myxospermic and trypanocarpic mechanisms was analysed separately, we found in the case of myxospermy that the model significatively explained almost 41% of the variance ($F_{3, 56} = 14.62$; $p < 0.0001$) indicating that soil erosion ($p = 0.0081$), but not climate dryness ($p = 0.8075$), significantly increased the proportion of myxospermic species in plots. However, the significant interaction term ($p = 0.0376$) indicates that the increase in the proportion of myxospermic species due to the effect of soil erosion is higher when climate dryness is greater. In the case of trypanocarpy, the model (Adjusted $R^2 = 0.3033$; $F_{3, 56} = 9.561$; $p < 0.0001$) shows that climate dryness does not have a significant effect ($p = 0.6645$) but that soil erosion increases the proportion of trypanocarpic species ($p = 0.0086$). However, again, the significant interaction term between climate dryness and soil erosion ($p = 0.0010$) indicates that the proportion of trypanocarpic species in the “cool&wet” end of the climate gradient, is a little higher in the “no erosion” plots than in the “severe erosion”, but that the contrary occurs in the “warm&dry” end of the climate gradient (see Table 1).

Table 1: Average and standard deviation of the number (sp) and percentage (%) of species with different dispersal and soil anchorage mechanisms in 20 m^2 plots and ant nest density per hectare (nests) in hillslopes for the different treatments (N = 15). “Terms in the model” gives information about factors that have significant effect on the response variable in the linear model (C= climate dryness, E= soil erosion).

		Total	“cool&wet”		“warm&dry”		Terms in the model
			“no erosion”	“severe erosion”	“no erosion”	“severe erosion”	
Atelechory	sp	26.3 ± 8.5	39.3 ± 3.0	24.5 ± 4.5	21.6 ± 3.9	20.0 ±3.8	C
	%	79.3 ± 4.5	79.2 ± 3.3	76.8 ± 4.3	81.0 ±5.8	81.3 ± 2.8	
Seed anchorage mechanisms	sp	9.8 ± 3.0	13.5 ± 1.4	10.6 ± 1.7	7.0 ± 2.0	8.2 ± 1.9	E
	%	30.1 ± 5.6	27.0 ± 3.1	33.5 ± 4.1	26.3 ± 5.3	33.5 ± 5.2	
Myxospermy	sp	8.4 ± 2.5	11.5 ± 1.5	8.6 ± 1.3	6.0 ± 1.6	8.6 ± 1.3	E, CxE
	%	26.0 ± 5.5	22.9 ± 3.0	29.3 ± 3.0	22.5 ± 4.7	31.4 ± 5.7	
Trypanocarpy	sp	0.8 ± 0.7	1.1 ± 0.5	1.3 ± 0.5	0.6 ± 0.7	0.3 ± 0.5	E, CxE
	%	2.4 ± 2.2	2.1 ± 1.0	4.2 ± 1.6	2.1 ± 2.6	1.1 ± 1.9	
Ant nest density	nests	100.2 ± 87.1	186.7 ± 96.1	36.6 ± 40.6	97.6 ± 89.1	81.8 ± 38.6	C, E, CxE

As hypothesized, both seed anchorage mechanisms (myxospermy and trypanocarpy together) and myxospermy were significantly and directly related to fine sand content and bulk density and also significantly but inversely related to water content (Table 2). This indicates that there is an increase in the proportion of species with anchoring mechanisms (mainly myxospermy) when physical soil properties related to runoff generation and water uptake worsen. Atelechory was positive and significatively related to fine sand content and inversely to water availability but not to bulk density (Table 2).

Table 2: Pearson correlation indices between the percentage of species with different dispersal and soil anchorage mechanisms and soil properties

	Fine sand content (%)	Water availability	Bulk density
Atelechory	0.2713*	-0.3233*	0.0169
Seed anchorage mechanisms	0.2753*	-0.2948*	0.3479**
Myxospermy	0.3277**	-0.4260***	0.3745**
Trypanocarpy	-0.1048	0.2708*	-0.0489

(p-value:n.s.<0.05; * 0.05-0.01; ** <0.01; N = 60).

Nests of ants collecting diaspores in the studied hillslopes were attributed mainly to the genus *Messor* (granivorous) and to a lesser extent to *Aphenogaster* (omnivorous). The total density of nests of these ants in hillslopes was highly variable (from 0 to 317.5 nests/ha, Table 1) and the linear model fitting our data (Adjusted R^2 = 0.2668; $F_{3, 24}$ = 4.276; p = 0.0149) showed that ant nest density significantly decrease because of climate dryness (p = 0.0295) and soil erosion (p = 0.0019), but the decrease of ant nest density because of soil erosion is higher in the “cool&wet” than in the “warm&dry” treatment (interaction term, p = 0.0287, Table 1).

4. Discussion

The proportion of species with diaspore anchorage mechanisms in our study area is similar, or even higher, to that found in desert and semi-desert areas of Africa (Van Rheede van Oudtshoorn and Van Rooyen 1999). At least 18.6% of the plant species in our study area and 17.0% of the species living in Namaqualand (Namibia) have anchorage mechanisms in their diaspores and 15.6% of the species in our study area, 15.7% in southeast Spain, 11.3% of the species in Namaqualand and 11.1% in northwest Africa are myxospermic (Van Rheede van Oudtshoorn and Van Rooyen 1999; Navarro et al. 2009). In relation to trypanocarpy, only 3.0% of the species in our study area are trypanocarpic, a similar value as in Namaqualand (5.7%) and in semi-desert areas of southeast Spain (<4%, Navarro et al. 2009). The proportion of species with short-distance dispersal syndromes (atelechory) was very high, approximately 80%, and is similar to that of desert areas of Israel (75.0%) but higher than that of Mediterranean and semi-desert areas of Israel (50.0%) and semiarid areas of Namaqualand (21.6%) (Ellner and Shmida 1981; Van Rheede van Oudtshoorn and Van Rooyen 1999). Interestingly, 100% of the myxospermic species also have short-distance dispersal mechanisms and 22% of atelechoric species are myxospermic. Ant nest density was also in the range of values reported for other semiarid shrubland (Diaz 1991; Azcárate and Peco 2003).

According to the prediction of the Ellner-Shmida’s hypothesis, soil erosion but not climate dryness, increased the proportion of species with anchoring mechanisms living on hillslopes in our study area. Similarly, and in agreement with our prediction, atelechory increased, although slightly, with climate dryness, but soil erosion did not affect it. These results and the correlations between the proportions of species having atelechoric or seed anchorage mechanisms with the studied soil properties strongly suggest that the need to resist seed removal by water runoff and to enhance the rate and the amount of water uptake by seeds are selective forces affecting the proportion of species with seed anchoring mechanisms, mainly myxospermy, in our study area. However, the significant correlation of atelechory with fine sand content (positive) and water availability (negative) and the fact that all myxospermic species have also atelechoric syndromes, supports the idea that soil degradation because of vegetation openness positively selects plant species with short-distance seed dispersal mechanisms (Ellner and Shmida 1981; Kefi et al. 2008; Pueyo et al. 2008) but that myxospermy should be a specific adaptation to soil erosion.

It is interesting to note that although the proportion of seed anchorage and myxospermic species are positively correlated with bulk density and bulk density increased with both, climate dryness and soil erosion, only soil erosion, but not climate dryness, positively affected the proportion of species with seed anchorage mechanisms and myxospermy. This can be explained by the fact that although bulk density decreased from the flat “warm&dry” plots to the flat “cool&wet” plots (García-Fayos and Bochet

2009), the low slope angle recorded in these areas (<5º) prevents water sheet flow from increasing in the same way. Therefore, plants of the flat “warm&dry” plots do not suffer from more intense erosion than their equivalents in the “cool&wet” area and consequently they do not need to increase mechanisms against seed removal by erosion.

Our results also shed some light on the different roles that myxospermy and trypanocarpy play as seed anchorage mechanisms, although the very low frequency of trypanocarpic species (<1.5±0.9 species/plot in average) precludes obtaining robust conclusions. Whereas almost all the conclusions about seed anchorage mechanisms are applicable to myxospermy, this is not the case for trypanocarpy. In fact, the percentage of trypanocarpic species per plot is only significantly correlated with water availability and this relationship is positive, unlike myxospermy. This reinforces the results from the linear model indicating that trypanocarpic species seem to be selected by soil erosion only in the wetter extreme of the climatic gradient, but that myxospermic species are selected positively in both extremes of our climate gradient, although more intensely in the “warm&dry” extreme.

It is unlikely that the interpretation of our results on seed anchorage and myxospermy was masked by the pressure exerted by harvester ants on plant species composition. Ant nest density was negatively related to soil erosion, especially in the “cool&wet” extreme of the gradient, probably because of the negative effect of soil erosion on the standing biomass and productivity. That is, the pattern of variation of the proportion of species with seed anchorage mechanisms and that of myxospermic species we found in our study was the opposite that it can be expected because of the pressure of harvester ants on plants. This does not mean that ant harvesting pressure cannot be a relevant force selecting anti-predatory mechanisms for seeds, such as myxospermy and trypanocarpy (see Schöning et al. 2004 and Engelbrecht and García-Fayos 2012 for recent reports on this issue), but it simply means that this pressure cannot explain the proportion of species with seed anchorage mechanisms in our study design.

Our results have implications for the controversies on the role of seed dispersal in buffering unpredictable small or medium-scale differences in patch quality in semiarid environments. It has been repeatedly suggested that atelechory and seed anchorage mechanisms contribute to the origin and maintenance of arid and semiarid patchy landscapes (Schurr et al. 2004; Kefi et al. 2008; Pueyo et al. 2008) but to date this assumption remains untested and needs to be directly verified. Siewert and Tielborger

(2010) found that in Mediterranean semiarid communities most species do not rely on seed dispersal mechanisms to buffer unpredictable small-scale differences in patch quality, but rather that the major contribution of plant species to population recruitment was through the investment in seed numbers and dormancy. In this context, investing in seed anchorage mechanisms, such as those analysed here, can be another way for plant species to survive in semiarid landscapes where soil erosion plays an important selection pressure.

In conclusion, our report is, to our knowledge, the first attempt to examine systematically the importance of various factors associated with the selection of plant species whose diaspores have anchoring mechanisms to ground. Although the role of myxospermy and trypanocarpy as mechanisms enhancing seed-soil contact had already been widely studied, it is the first time that a direct relation of soil erosion with the proportion of myxospermy and trypanocarpy in plant communities has been shown, supporting the hypothesis that anchorage mechanisms in diaspores play a role shaping species composition in communities affected by intense soil erosion (see also Bochet et al. 2009 and García-Fayos et al. 2010). The correlative approach of our study and the complex nature of the explanatory characteristics used make it difficult to interpret unambiguously the ultimate causes behind the observed associations. Nevertheless, the patterns that we detected support the idea that in water-limited environments anchorage mechanisms can be a way for seeds to overcome the direct effects of soil erosion -seed removal- and the negative effects of soil erosion on soil properties related to water uptake by seeds, an important limiting factor for plant establishment in such environments (García-Fayos et al. 2000; Bochet et al. 2009; Moreno-de las Heras et al. 2011).

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Mucilage secretion by seeds doubles the chance to escape ant removal

CHAPTER 3

Abstract

Post-dispersal seed predation is a risk for plants in semiarid environments, leading to strategies to protect their propagules from seed collection by animals. In this study, we evaluated the importance of mucilage secretion in seeds as a mechanism to reduce seed collection by ants. We selected three Mediterranean species with strong mucilage secretion on their seeds which become sticky upon wetting. Seeds of *Rosmarinus officinalis*, *Fumana ericoides* and *Fumana thymifolia* were exposed to ants and survival was compared between dry loose seeds and seeds glued to the soil with previously secreted mucilage. The study site was in the Sierra Calderona, 25 km north of Valencia (Spain).

The ant-plant interaction was analysed by scrutinizing seed collection by ants and by analysing the waste piles of ant nests. To test survival, groups of 10 seeds were placed on the ground. Each group consisted of 5 control (dry) and 5 mucilaginous seeds (previously mucilage secreted) and was covered by the cover of a Petri dish modified to permit only the entry of ants. Seeds were inspected weekly for seed disappearance and the survival function (Kaplan-Meier estimator –KM) was calculated.

Seeds of the target species were important food items for ants and were actively collected, and more than 50% of the experimental seeds that were glued to the ground with their own mucilage survived at the end of the study period but only 0-20% of the control seeds survived after the same time of exposure. The implications for plant establishment of these findings are discussed.

1 Introduction

In semiarid environments, post-dispersal seed predation is a frequent risk and has the potential to influence plant populations and community dynamics (Louda 1989; Davidson 1993; Hulme 1996, 1998; Crawley 2000; Azcárate and Peco 2007). An effective strategy to escape massive seed collection can be of crucial importance for plant species in order to maintain their population densities above extinction thresholds.

Seeds can be consumed by mammals, birds and insects (Janzen 1971; Crawley 2000; Kelt et al. 2004), but in some semi-arid and arid ecosystems granivorous ants have the strongest impact on post-dispersal predation relative to other animal groups (Mares and Rosenzweig 1978; Morton 1985; Kerley 1991; Predavec 1997; Hulme 1998; Lopez de Casenave et al. 1998). Ants have a strong influence because of their ability to search on large areas for food and also the great number of seeds they can collect in a season (Hölldobler and Wilson 1990). For some plant species it has been reported that ants collect more than 85% of available seeds, resulting in almost 70% of seeds effectively lost by predation (Retana et al. 2004).

Ants tend to collect a wide range of seeds depending on their availability (Hobbs 1985), but seed choice is also influenced by specific seed attributes, such as size, weight and structure (Willott et al. 2000; Azcárate et al. 2005). Some seeds are therefore strongly collected and strategies to escape massive seed collection in these species could be especially effective.

Plants have developed mechanisms to escape from massive seed collection by animals, such as mechanical or chemical defences, seed burial in the soil (Crawley 2000; Hulme and Benkman 2002; Schöning et al. 2004) or even seed dispersal by animals through rewarding with the fruit pulp or an elaiosome whereat the seed stays intact (Herrera 2002; Giladi 2006). Diaspores of many plants inhabiting semiarid habitats secrete a gel of polysaccharides around the seed or fruit coat which becomes sticky once they come in contact with water, getting them adhered to the soil upon drying (myxospermy) (Grubert 1974; Werker 1997). This hydrophilic, pectinaceous mucilage is deposited in the apoplast of epidermal cells in a compressed form during differentiation of the seed coat or pericarp, and is released on hydration to form a water-containing, gel-like capsule surrounding the seed (Western 2012). Once dried up it glues the seed to the ground with a thin and strong layer and can rehydrate completely with the next rain or dew, drying and swelling for many times with almost no loss in volume. Rain and moistened soil are enough to release mucilage secretion, but Huang et al. (2004; 2008) have shown that experimental and natural dew at nights is enough for seeds to secrete mucilage. This adherence to the ground has been suggested as a mechanism to reduce diaspore removal by erosion on slopes (García-Fayos et al. 2010) having the potential to act as a mechanism to escape from seed removal by ants. Seed harvesting by ants in the Negev Desert (Israel) was effectively slowed down and seed collection of *Salvia columbaria* in California (USA) was strongly reduced because of mucilage secretion (Fuller and Hay 1983; Gutterman and Shem-Tov 1997). Both studies were done over very short time periods, between two days and a week, so the long-term efficacy of the mucilage was untested.

In this study we tested the hypothesis that myxospermy effectively reduces seed removal by ants in the medium timeframe using a set of plant species inhabiting open, semiarid Mediterranean shrubland. Our objective was to test: a) if seeds of the experimental plant species are of interest to ants; b) if survival of seeds glued to the soil by mucilage is higher when exposed to harvesting ants than dry and loose seeds of these species; c) the implications of these findings for plant establishment in semiarid Mediterranean shrubland.

2 Methods

2.1 Study area

The study site is located in the municipality of Serra (39° 39' N, 0° 29' W) in Parque Natural de la Sierra Calderona, about 25 km north from Valencia (Spain) at 250 m altitude. The vegetation consists of open *Pinus halepensis* forest with a very rich layer of shrubs (*Rosmarinus officinalis*, *Erica multiflora*, *Cistus albidus*, *Rhamnus lycioides*, *Pistacia lentiscus*, *Thymus vulgaris*, *Helianthemum spp.* and *Fumana spp.*) and perennial grasses (*Stipa tenacissima* and *Brachypodium retusum*) reaching 50% of soil cover in average (Andreu et al. 1998). Soils are loam and sandy-loam, rich in calcium carbonate (>40%), poor in organic matter (<5%) highly compacted and near of 3% of bare soil is covered by crust (Andreu et al. 1998). Mean annual temperature is 17.4 ºC and the mean annual rainfall 467 mm (García-Fayos and Gasque 2006). The total study area spans about 1.5-2 ha and has very little human influence, with scarce agriculture in the surroundings and no exposure to grazing for decades.

2.2 Plant species

We use fruits of rosemary (*Rosmarinus officinalis*, Lamiaceae) and seeds of needle sun roses (*Fumana ericoides* and *F. thymifolia*, Cistaceae) based on two criteria: their high amount of seed production or large seed size both in combination with strong mucilage production. *R. officinalis* is a 0.5 to 1.5 m tall shrub widely distributed in the Mediterranean basin which produces large quantities of fruits. Fruits are soft nutlets (0.46 mg ± 0.024 mg, Lloret et al. 1999) that are an important food source for granivorous ants. For simplicity, we hereafter refer to them as seeds. *Fumana ericoides* and *F. thymifolia* are small sized shrubs, up to 0.4 m tall (Güemes and Molero 1993). Seeds are relatively large (2.44 mg ± 0.054 mg and 1.08 mg ± 0.023 mg respectively, Lloret et al. 1999) but they are produced in lower quantities than in *R. officinalis*. Despite their hard coats, seeds of *Fumana* are also strongly collected by granivorous ants (Arnan et al. 2010, author’s personal observations). The diaspores of the three species become covered with thick mucilage once they come in contact with water, which takes about one to five minutes to fully secrete. Dew has been observed to release the secretion of mucilage in these species (author's personal observation).

Seeds of all three species (or the whole calyx in *R. officinalis*) are detached from the mother plant by gravity or by wind, rain or animal disturbance (Güemes and Molero 1993 and authors’ personal observations), sometimes followed by secondary seed dispersal by ants (myrmecochory; Bouman et al. 1992; Arnan et al. 2010). In some occasions, we observed ants collecting fruits, seeds and ripe calices directly from the plants.

2.3 Ant species and their interaction with the target plant species

The research area displayed an average ant nest density of 175 nests per hectare (García-Fayos and Gasque 2006). Six different ant species are present in the study site: *Messor bouvieri*, *M. capitatus*, *Aphaenogaster iberica*, *Formica subrufa*, *Tapinoma nigerrimum* and *Camponotus sylvaticus*. Of these species, the genus *Messor* (47.8% of the ant nests in the study area) is the only reputed granivorous ant. *A. iberica* (13%) is ambiguously considered as granivorous (Azcárate and Peco 2011) and omnivorous (Rey et al. 2002), while *F. subrufa* (8.6%) and *T. nigerrimum* (26%) are considered to be nectarivorous and omnivorous (Cavia 1989; Cerdá et al. 1989).

Camponotus sylvaticus (4.3%) is recognized as a nectarivorous ant (Retana et al. 1988).

All the ant species except *C. sylvaticus* and *T. nigerrimum* were observed carrying seeds during the observations, indicating that granivorous and omnivorous ant species were actively searching for seeds in the whole area.

To properly interpret the results of our seed survival experiment we needed to know the strength of the interaction between the ants and the target plant species in our study site. In 2009 we selected and marked 15 nests of ants of *Messor bouvieri*, *M. capitatus*, *Formica subrufa* and *Aphaenogaster iberica*, the most frequent ant species collecting seeds in the experimental area, and marked one representative plant of *R. officinalis*, *F. ericoides* and *F. thymifolia* close to each ant nest. Over the time that the seed removal experiment was performed, we assessed the fruiting status of each plant every one or two weeks and estimated the amount of seeds available for ants. Additionally, we surveyed seed collection by ants in 10 of the 15 marked ant nests to determine the importance of seeds of the target species as a food source. For this, we counted the number of items that ants transported to individual nests during 10 minutes periods through the study period, with a total of 32 counts irregularly distributed among the 10 ant nests in function of the coincidence of observation periods with ant activity. Also we collected the entire waste pile from 14 of the 15 marked nests at different times along the experiment. To analyse the content of the waste piles, about 5% of dry weight was evaluated completely after mixing the entire waste pile and the relative content of the target plant species analysed. The content of the waste pile was then pulled apart visually under the binocular microscope. The separated material was weighted on a precision balance and the relative content of the seeds evaluated (seed weight/ total weight of the waste pile; where total weight includes all vegetal, animal and mineral materials). The seeds of the target species in our experiment were counted to species level, while the remaining seeds were recorded to the genus or family level when possible.

2.4 Seed removal experiments and seed survival analysis

To evaluate the importance of mucilage secretion as a mechanism to escape seed removal by granivorous ants, we performed experiments on ant removal of loose vs. glued seeds (by their own mucilage) of *R. officinalis*, *F. ericoides* and *F. thymifolia*.

In the spring of 2009, groups of 10 experimental seeds were placed within a radius of 5 m but not closer than 50 cm from marked ant nests. We placed one group for each studied plant species in the surroundings of ant nests, with a total of 15 replicates per plant species. Half of the seeds in the groups remained dry and loose (control seeds) and the other half of the seeds were placed in distilled water for 20 minutes until full mucilage secretion was reached. We then placed 5 control seeds and 5 moist (mucilaginous) seeds on a flattened patch of bare soil. Petri dish covers (9 cm Ø) were then carefully placed on top and secured with a stone to avoid its displacement. Petri dish covers were provided of two small entrance holes in the side (1 x 1 cm) to permit only the entry of ants. Mucilaginous seeds became glued to the soil surface within 1 h after putting them on the ground. Once mucilaginous seeds had become glued to the ground, they could only be detached when the soil around it was disturbed by animals.

A total of 75 dry and loose (control seeds) and 75 mucilaginous seeds per species were used in the experiment and seed survival was checked every week. Groups of seeds were replaced as soon as control seeds changed the condition from loose to glued to the ground, because heavy rain, or when Petri dishes got destroyed. The experiments ran from the start to the end of the seed dispersal season of each shrub species (35 days in *R. officinalis*, 84 days in *F. thymifolia* and 91 days in *F. ericoides*).

The fate of control and glued seeds were individually followed through the experiments and values of 0 and 1 were assigned to represent seed survival and seed removal, respectively. In all trials, the survival time, which served as the response variable, was considered to be the date of the latest survey that the seed was detected.

The Kaplan-Meier estimator (KM) of the survival function was calculated for each of the different seed treatments and plant species. We then checked for differences in survival between the two treatments using the log-rank test (Mantel 1966; Cox 1972). The statistical analysis was made in R statistical package (V. 2.12.2).

3 Results

3.1 Ant species and their interaction with the target plant species

From the inspection we made of items that granivorous ants carried to their nests, we can confirm that ants searched and collected large numbers of seeds of many plant species in the study area (a total of 2206 counted seeds during the observation times during the study period). About 42.5% of all observed items were diaspores of the species *R. officinalis, F. ericoides* and *F. thymifolia* (1109 items in 320 minutes) and

75.5% of the dry weight (comparing only the plant remains without soil) in the waste piles of ant nests belonged to propagules of these species.

Large numbers of ripe calices of *R. officinalis* were collected by ants in the first half of April, when nearly 25% of the items that ants transported to nests were seeds or entire calices of this species (Figure 1a). In the case of *F. ericoides*, ants were increasingly interested in seeds of this species following its commencement of its fruiting period but never exceeded 10% of items that seeds transported to the nests (Figure 1b). For *F. thymifolia,* ants were found transporting high amounts of seeds of this species at the beginning of the fruiting period, counting to up 20% of the items seeds carried, and subsequently decreased (Figure 1c).

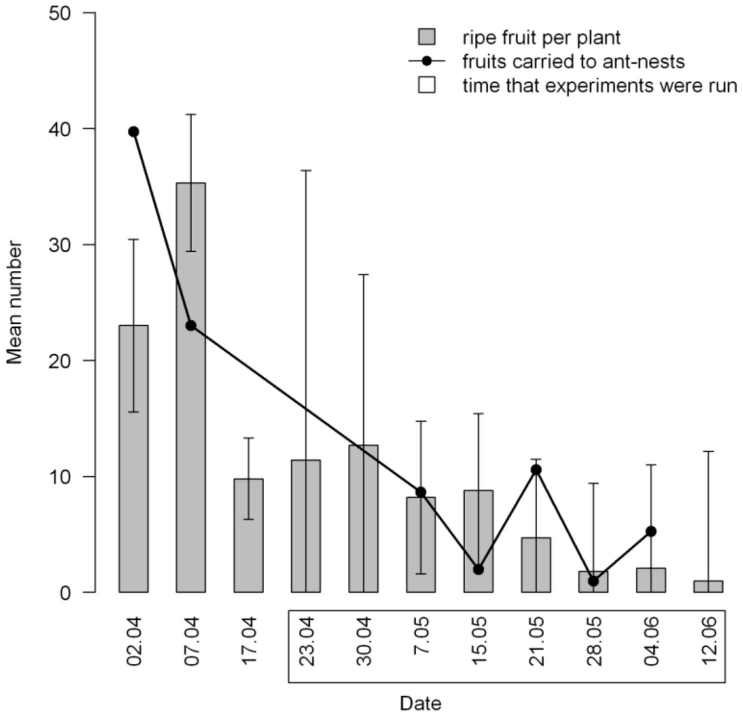

Figure 1a: Ripe fruit of *Rosmarinus officinalis* (*R*. *officinalis* with 1-4 seeds per fruit) and seed availability scores in an average of 15 plants, with mean number of seeds carried by ants to nests in four counts of 10 minutes and time that fruits and seeds were offered in trial experiments to ants over the studied time period.

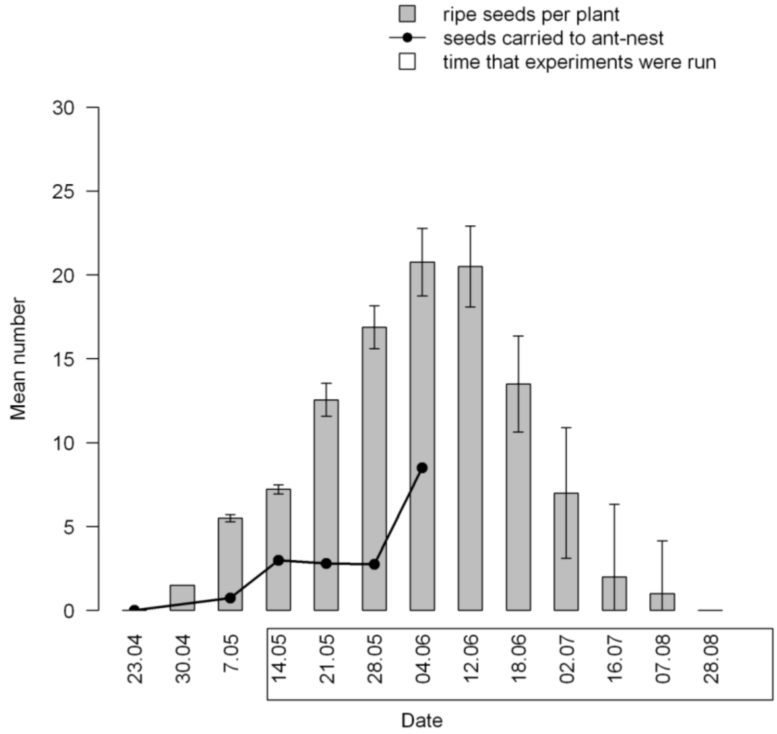

Figure 1b: Ripe fruit of *Fumana ericoides* and seed availability scores in an average of 15 plants, with mean number of seeds carried by ants to nests in four counts of 10 minutes and time that fruits and seeds were offered in trial experiments to ants over the studied time period.

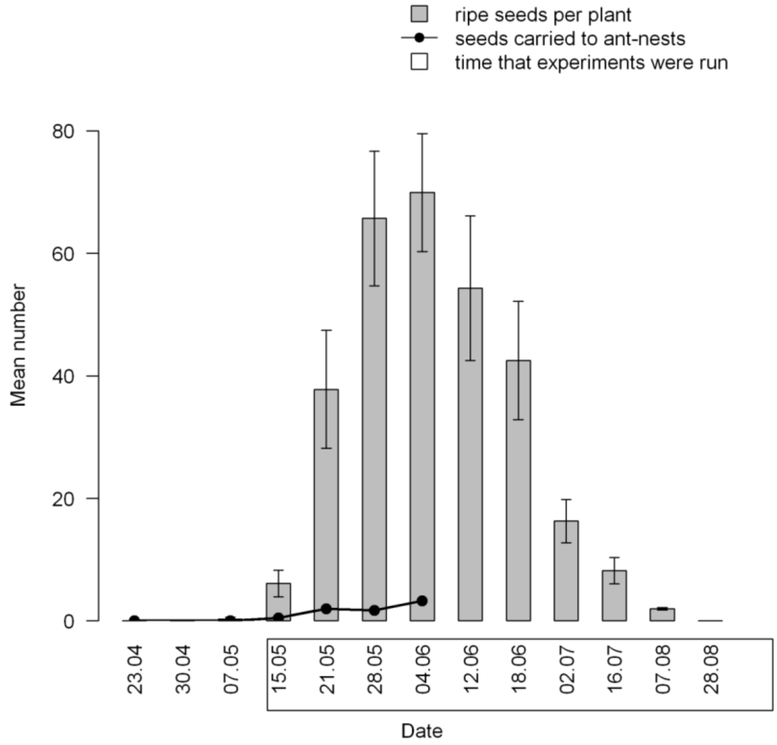

Figure 1c: Ripe fruit of *Fumana thymifolia* and seed availability scores in an average of 15 plants, with mean number of seeds carried by ants to nests in four counts of 10 minutes and time that fruits and seeds were offered in trial experiments to ants over the studied time period.

The highest proportion of items in the waste piles of ant nests were entire calices of fruits of Cistaceae, calices and seeds of *R. officinalis*, calices of *Thymus vulgaris*, and seeds of *Stipa tenacissima* plus different species of *Fumana* (Figure 2). Remains of other diaspores found in the waste pile were that of *Pistacia lentiscus*, *Teucrium* sp. and *Thessium humifusum*. Other vegetative plant components and reproductive remains of unknown species were present but are not displayed in the figures.

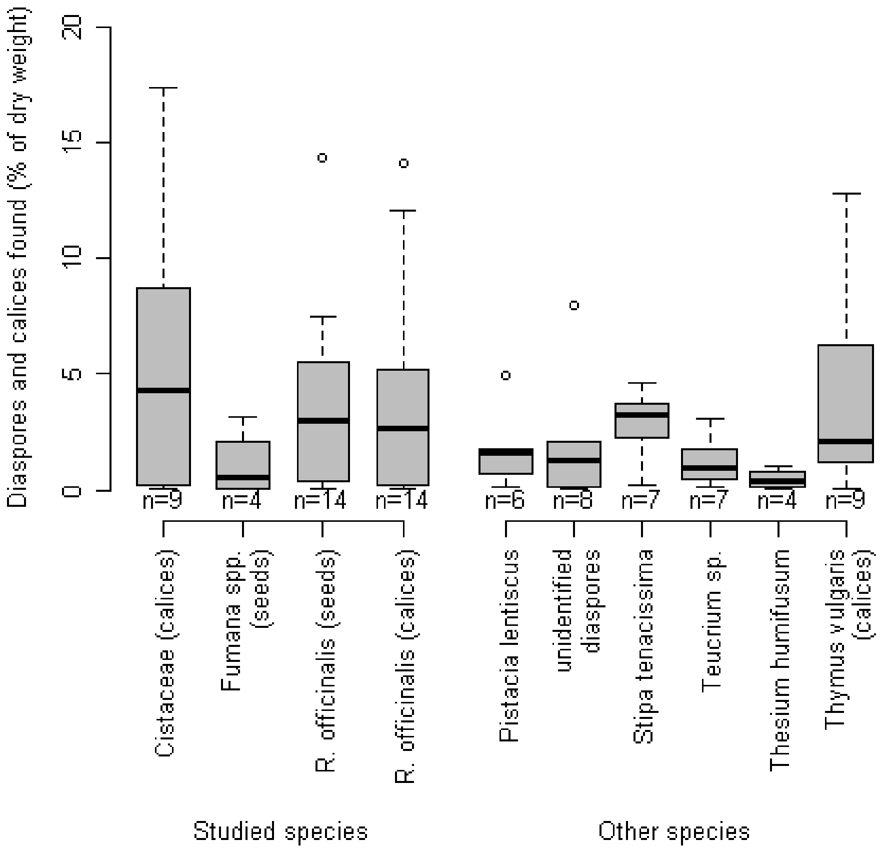

Figure 2: Boxplots of the relative weight of remains of the target species in the waste piles of 14 ant nests in the area where the experiments on seed removal by ants were performed. Values were calculated from dry weight data. Only reproductive remains of plants were displayed (n: number of ant nests where an item class was found). The boxplot displays the smallest and largest value as well as the first quartile, the median and the third quartile.

3.2 Seed removal experiments and seed survival analysis

Experimental seeds of *R. officinalis* were offered to ants at the end of the fruiting period of this species (Figure 1a). In the case of *F. ericoides*, experimental seeds were offered to ants from the third week that ripe fruits were observed in the field (Figure 1b) and in *F. thymifolia* the experiment started in the first week in which ripe fruits were counted (Figure 1c).

Seeds that were glued to the ground with their own mucilage survived significantly longer to removal by ants than control (dry and loose) seeds in all three studied species (Figures 3a, b and c) and the differences were significant in all the species.

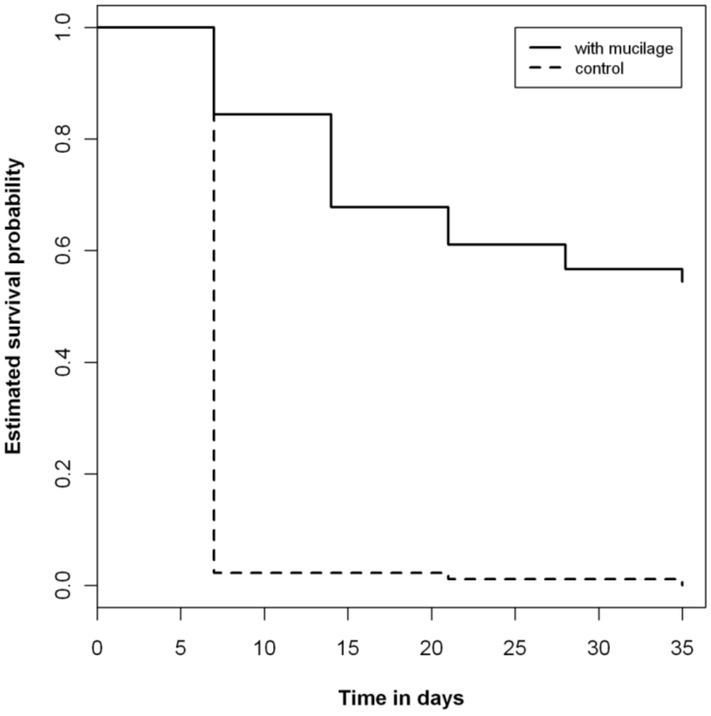

Figure 3a: Estimated survival probability along time of glued and control seeds based on the computed values of the Kaplan-Meier estimator for *Rosmarinus officinalis.*

Control seeds of *R. officinalis* were predated strongly the first week that the experiment started and they reached a survival probability of 2% after 7 days and 0%

after 35 days. Glued seeds were significantly less predated, with a survival probability of 84% after 7 days and still 54% of probability of survival after 35 days of exposure (X^2=125, p <0.00001, Figure 3a).

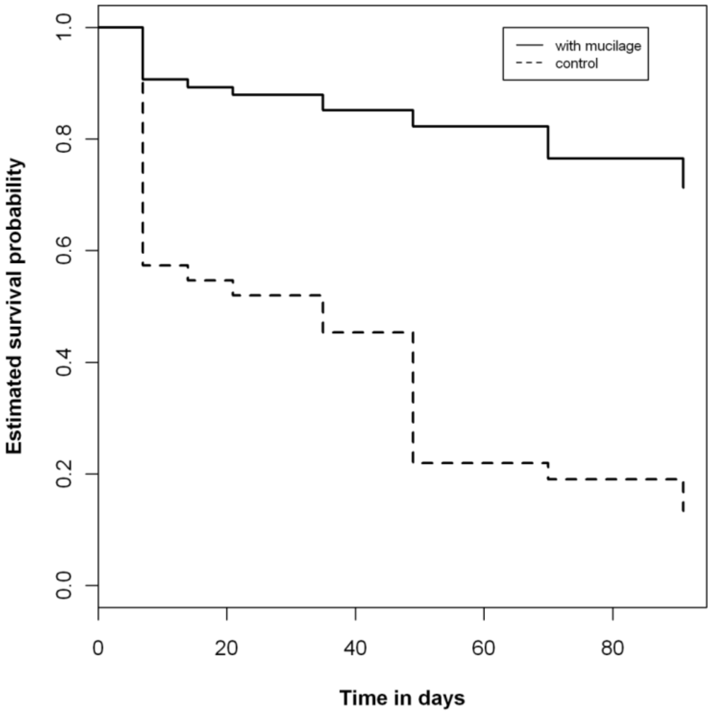

Figure 3b: Estimated survival probability along time of glued and control seeds based on the computed values of the Kaplan-Meier estimator for *Fumana ericoides*.

Control seeds of *F. ericoides* reached a 57% survival probability after 7 days and a minimum of 13% after 91 days, while glued seeds showed a significantly higher survival probability, with 90% of seeds surviving after 7 days and a 71% survival probability after 91 days (X^2=55, p< 0.0005, Figure 3b).

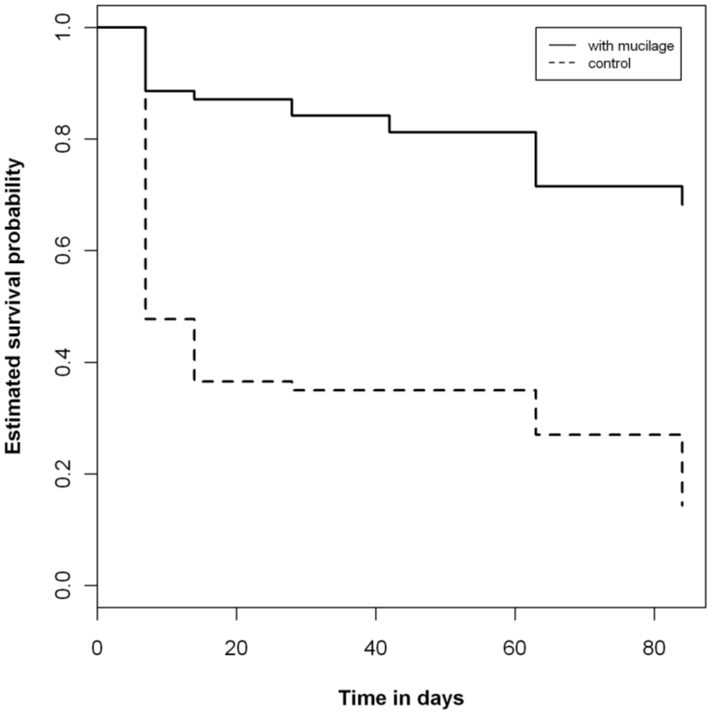

Figure 3c: Estimated survival probability along time of glued and control seeds based on the computed values of the Kaplan-Meier estimator for *Fumana thymifolia*.

Seeds of *F. thymifolia* glued to the ground had a survival probability of 88% after 7 days and remained very high, 68%, after 84 days of exposure. Control seeds reached a probability of survival of 47% after 7 days and a minimum 14% after 84 days of exposure. Differences in seed survival were significant (X^2= 44.6, $p<0.00001$, Figure 3c).

4 Discussion

Our experiments showed that seeds of the studied plant species are heavily collected by ants and that mucilage secretion of these diaspores strongly reduced ant removal, thus favouring a longer persistence in the field. These findings support the idea that granivory by ants is an important factor in seed survival in the study area and that the escape mechanism we here addressed may be relevant for the target plant species.

Inspection of seeds carried by ants and waste piles indicated that seeds of all three species were items of interest for ants. The high proportion of remains of *R. officinalis* in the waste piles and the relatively low proportion of remains of *Fumana* species is consistent with the survival curve of *R. officinalis*, which is removed to a greater extent than the other species (Figures 3a, b and c), thus seeming to be of higher interest to ants. Diet and seasonal pattern of harvesting may be explained by relative seed abundance (Briese and Macauley 1981; Hobbs 1985; Willott et al. 2000) and other factors, such as seed coat strength, which could influence ant's preferences. This could explain why seeds of *Rosmarinus* are more likely to be eaten than stronger seeds of *Fumana* species (Rodgerson 1998). Still, seeds of the family Cistaceae are of high interest as a food source and are intensely collected in the Mediterranean ecosystem we studied (Bastida and Talavera 2002; Bastida et al. 2009).

More than 50% of the glued seeds still survived at the end of the experiments but only 0-20% of the control seeds survived at that time and this strong antipredatory effect that mucilage has on seed removal is similarly effective in all species. In natural conditions mucilage secretes once the seed become moistened (Werker 1997) which occurs during rain, fog or dew (see Material and Methods) and seeds adhere strongly to the soil after drying, thus preventing further removal. Heavy rain events are usually scarce in summer under Mediterranean climate conditions when seeds are released from plants (4.5 days in average in our study area; data from the 2004–2011 period of the Valencia and Castellón meteorological stations, provided by the National Meteorology Agency, Ministerio de Medio Ambiente, http:/www.aemet.es/) but dew, fog and light rain are more frequent meteorological events in Mediterranean semiarid conditions. Morning dew can provide significant amounts of water in dry Mediterranean areas (Kosmas et al. 1998; Kidron 1999; Agam and Berliner 2006), and it frequently occurs during the summer; up to 20 days in late summer (Baier 1966). Summer is usually the season with the lowest fog water yield but still it can make up an important amount of moisture in comparison with rainfall events, occurring over 25-38% of the days without rain in the summer months (Estrela et al. 2008).

Granivorous ants can also act as seed dispersers as they accidentally abandon viable seeds on the way to the nest or deposit them in other places that the waste piles (Wolff and Debussche 1999; Retana et al. 2004). Therefore, the view that granivorous ants only harm seeds has been frequently criticized. Ant removal can have beneficial and detrimental effects, destroying most of the seeds but selectively benefitting some of them (Levey and Byrne 1993; Boyd 1996). In areas where most seeds are consumed, these exceptions can have significant influence on vegetation composition (Dean and Yeaton 1993; Arnan et al. 2010). It has been reported that up to 16.4% of the harvested seeds are lost on the track by *Messor bouvieri* and that these seeds were not recovered afterwards, therefore being effectively dispersed (Retana et al. 2004). Ant-mediated dispersal has been previously reported for *R. officinalis* and *F. ericoides* (Bouman et al. 1992; Arnan et al. 2010), suggesting the importance of ant removal of seeds for the colonization of open patches. However, some ant species, such as *M. barbarus*, repeatedly find and recover seeds lost on the way to the nest (Detrain and Tasse 2000; Schöning et al. 2004). Whatever the case is, lost seeds on the track to the ant nests can increase their chance to survive if they get glued to the ground, escaping further predation and runoff. This is possible when it coincides with a rainfall event, or more probably, with strong morning dew or fog in summer. This escape strategy seems especially important for *R. officinalis* seeds, as their seeds are heavily predated before and during seed ripening. Often, ants even cut whole ripe calyxes directly from the plant to transport them to the nest, making the loss of single seeds on the track highly probable, as completely ripe seeds easily fall out of the calyx when it is moved (author's personal observation).

At the end of August we removed the Petri dishes covering the seeds of *F. ericoides* and *F. thymifolia* (only glued seeds remained under them) and followed the fate of the seeds. After the first rain in September we observed that 50% and 40.8% of the seeds of *F. ericoides* and *F. thymifolia* respectively germinated, in accordance with time of germination reported from field observations of these species and that of *R. officinalis* (Lloret 1998; De Luís et al. 2005). This observation and the results of our experiment of seed removal support the idea that myxospermy plays an important role in determine the spatial pattern of seedling recruitment of these species. So, Arnan et al. (2010) found that overall, 88% of all patches newly occupied by *F. ericoides* along three years were <0.5 m from adult individuals, which agrees with the primary seed dispersal distances of this species. Similarly, López et al. (2003) found that for *R. officinalis*, most of the new recruitments during two years were located within 1.75 m around the mother plants. For *F. thymifolia*, Jump et al. (2009) studying the within-population genetic structure of this species using amplified fragment length polymorphism (AFLP), found very limited seed dispersal within the population.

In synthesis, we found that diaspores of the studied plant species were actively searched and collected by ants. Those diaspores that were glued to the ground due to their own mucilage secretion doubled the chance of surviving ant collection in comparison to the control seeds after several months, thus increasing their chance to establish and providing a mechanism to explain the recruitment patterns of these shrubs.

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Phylogeny, biogeography and morphological ancestral character reconstruction in the Mediterranean genus *Fumana* (Cistaceae)

CHAPTER 4

Abstract

The genus *Fumana* is one of the most diverse and least studied genera of the Cistaceae family. To date, there are 21 recognized species with high morphological diversity, divided into three subgenera (*Fumana*, *Fumanopsis* and *Pomelina*). We used three molecular markers with coding (*matK*) and spacer (*trnT-L*) sequences of plastid and nucleic DNA (ITS) to reconstruct the phylogeny of 19 *Fumana* species (using Bayesian Inference, Maximum Parsimony and Maximum Likelihood analysis) and to estimate divergence times (using Bayesian analysis). Phylogenetic relationships based on the plastid markers confirmed the monophyly of the genus. Results do not support the traditionally established infrageneric divisions, but confirm the presence of two main groups of species. Each clade clusters species differing in vegetative and reproductive characters and having been formerly grouped in three subgenera (*Fumana*, *Fumanopsis* and *Pomelina*). However, none of the clades clustered species exclusively from one of the recognized subgenera. Given the impossibility of defining morphologic characters which are common to all species of every clade we reject all infragenetic divisions and discard the existence of three subgenera. Nevertheless, phylogenetic analysis supports the taxonomic delimitation of nine species, frequently discussed in recent years. Significant ancestral character states were found in five cases (leaf form and leaf margin, glandular trichomes, diaspore and seed mucilage secretion), suggesting an adaptation to the Mediterranean environment and climate. Divergence times of *Fumana* date to around 16.97 Ma ago (24-10 Ma), with the divergence of major clades between the Middle and Late Miocene (15.61-8.8 Ma). Area analysis reconstruction suggests *Fumana* has a western Mediterranean ancestor, with this region representing the main centre of diversification, and the eastern Mediterranean representing a secondary diversification zone.

1 Introduction

The Mediterranean Basin is known for having many plant species and endemisms, making it a major hotspot for global biodiversity (Médail and Quezel, 1999; Sauquet et al., 2009). The contemporary flora in the Basin is the result of tectonic movements and climatic oscillations acting at different spatial and temporal levels (Thompson, 2005), which led to complex patterns of connection-isolation between territories (Rosenbaum et al., 2002; Meulenkamp and Sissingh, 2003; Ree and Sanmartín, 2009). Also, the formation of land bridges during the Oligocene and Miocene led to biotic expansions across the Mediterranean (Oosterbroek and Arntzen, 1992; Salvo et al., 2010) and we can still recognize biogeographical links between some plant clades that diversified in the western Mediterranean and related eastern Mediterranean taxa (Médail and Diadema, 2009; Jabbour and Renner, 2011).

Insights into the evolutionary path of a plant family can be inferred from the evolution of morphological characters, based on phylogenetic analysis. Certain patterns of character evolution are typically in Mediterranean plant families and may indicate specific adaptations to climatic changes (Ackerly et al., 2002; Ackerly, 2004). Accordingly, leaf size and shape and trichome density have been viewed as adaptations to the increasing dryness and seasonality of the Mediterranean region (Fiz-Palacios et al., 2006; Guzmán et al., 2009; Guzmán and Vargas, 2009b; Turini et al., 2010). Recent years have seen a growing interest in both spatial and temporal patterns of diversification and speciation of plant groups in the Mediterranean region. Research has also attempted to understand the changes in the morphological characters that have marked the course of evolution of these groups (Guzmán and Vargas, 2005; Fiz-Palacios et al., 2006; Guzmán et al., 2009; Salvo et al., 2010). Despite this, there are very few groups of Mediterranean plants that have been studied from both perspectives (Guzmán and Vargas, 2005; Galbany-Casals et al., 2009; Guzmán and Vargas, 2009a; Pérez-Gutiérrez et al., 2012).

Cistaceae is one of the most representative plant families of the Mediterranean region. The Cistaceae family, consisting of eight genera, five in the Old World (*Cistus* L., *Halimium* (Dunal) Spach, *Helianthemum* Mill., *Tuberaria* (Dunal) Spach and *Fumana* (Dunal) Spach) and three in the New World (*Crocanthemum* Spach, *Hudsonia* L. and *Lechea* L.), had the Mediterranean as the main differentiation centre (Arrington and Kubitzki, 2003; Guzmán and Vargas, 2009a). Phylogenetic hypotheses about the family, based on molecular and morphological analyses, indicate the isolated position of the *Fumana* genus at the base of Cistaceae (Ukraintseva, 1993; Nandi, 1998a; b; Guzmán and Vargas, 2009a). The early divergence of this lineage, which seemed to have taken place in the Miocene (18.51-10.7 Ma), contrasts with that of most of the genera in the Old World, which took place in the Pliocene (4.22-5.30 clade *Tuberaria-Halimium-Cistus*; Guzmán and Vargas, 2009a). *Fumana* is a circum-Mediterranean genus and one of the most diverse and least studied genera of the family, and therefore represents an interesting plant system to study the process of plant evolution in the Mediterranean Basin.

The morphological differentiation of the genus *Fumana* from the family of the Cistaceae was mainly based on the presence of a whorl of sterile stamens, and anatropous ovules arrangement (Spach, 1836a; b). Studies on morphological characteristics of reproductive and vegetative traits (Spach, 1836b; Willkomm, 1856; Grosser, 1903; Janchen, 1920; Jean and Pons, 1963; Güemes and Molero, 1993) have led to diverse proposals on the infrageneric organization that have been recognized to date. *Fumana* has been divided into three genera or subgenera (*Fumana*, *Fumanopsis* Pomel and *Pomelina* (Maire) Güemes & Raynaud) based, principally, on the reproductive characters: inflorescence, stamens, ovules, pollen and seeds (Pomel, 1860; Janchen, 1920; Raynaud, 1992; Güemes and Molero, 1993). Three species of *Fumana*, belonging to each of these subgenera respectively, were included in a molecular-based phylogeny of the family Cistaceae giving rise to reasonable doubt about the independence of the subgenus *Pomelina*, which should be integrated in the subgenus *Fumana* (Guzmán and Vargas, 2009a).

To date, there are 21 recognized species of *Fumana* which have an almost exclusively circum-Mediterranean distribution with two core diversification centres, one in the western region, principally in the Iberian Peninsula, and one in the eastern region, located in the Peninsula of Anatolia. The genus is distributed north to south, from the Anti-Atlas in southern Morocco and Algeria (along 30°N parallel) to the island of Godland (located in the parallel 57°N); and west to east, from Agadir (in the meridian 9°W) to the Urals (60°E meridian) (Grosser, 1903; Janchen, 1920; Janchen, 1925). Unlike other Cistaceae (*Cistus* and *Helianthemum*), *Fumana* is poorly represented in the Mediterranean islands, and missing in the eastern Atlantic oceanic islands (Canary Islands, Azores, Madeira). Its distribution covers four biogeographic regions: Mediterranean, Irano-Turanian, Circumboreal and Saharo-Arabic. The Mediterranean region has been proposed as the main centre of diversification, especially the Iberian Peninsula, with 18 species, of which six are endemic to this region. The Irano-Turanian region, especially the Anatolian peninsula, has been proposed as a secondary centre of diversification, with 10 species of which three are endemic. There are no exclusive species inhabiting others biogeographic regions. Only one species (*F. procumbens* (Dunal) Gren. & Godr.) reaches the Circumboreal region, and three species (*F. arabica* (L.) Spach, *F. laevis* (Cav.) Pau, and *F. thymifolia* (L.)Spach ex Webb) extend to the Saharo-Arabic region (Coode, 1965; Güemes and Molero, 1993).

Despite interest in understanding the evolution of the Cistaceae in the Mediterranean region, the evolutionary history of the genus *Fumana* has never been examined from a phylogenetic viewpoint or within a biogeographic context. Although the morphological character of these species is very well known, their role as possible adaptations and their ancestral states have not been established and analysis of this type could shed light on the formerly established subdivisions of the genus. Therefore to investigate the pattern of evolution in the genus *Fumana*, we have adopted an integrative approach, including a detailed phylogenetic study based on two plastids (*matK, trnT-L*) and one nuclear (ITS) region of 19 of the 21 recognized species. Our results provide a molecular dating of the phylogenetic tree, a biogeographic analysis and a character-state reconstruction analysis.

2 Materials and Methods

2.1 Species sampling

A total of 55 *Fumana* accessions, representing 19 of the 21 species currently recognized (Coode, 1965; Heywood, 1968; Greuter et al., 1984; Güemes and Molero, 1993; Güemes, 1999) were sampled for the study (Table 2). *Fumana grandiflora* Jaub.& Spach and *F. oligosperma* Boiss.& Kotschy could not be sampled as neither species has been collected since their first description in the 19th century, and DNA extraction from the original herbarium collection was not possible. Species were represented by more than one population, with the exception of *F. fontqueri* Güemes, *F. lacidulemiensis* Güemes and *F. trisperma* Hub.-Mor. & Reese, because each has only one known population. The populations were sampled throughout the geographic range of each species according to (Güemes and Molero, 1993; 2002) for the western Mediterranean species, to Coode (1965), for the eastern species, and Heywood (1968) for the north Mediterranean species. Based on previous phylogenetic studies (Dayanandan et al., 1999; Guzmán and Vargas, 2009a), accessions from the related genera *Hopea* and *Neobalanocarpus* (Dipterocarpaceae), and *Cistus* (Table 2) were used as outgroups.

2.2 DNA extraction and amplification

DNA was extracted from freshly collected leaves, subsequently dried and stored in silica gel, or from leaves taken from herbarium vouchers (Table 2). DNA was extracted with the Speedtools Plant DNA extraction Kit (Biotools, Spain) following the manufacturer´s protocol but modifying the lysis step by adding 2-Mercaptoethanol and PVP till reaching a final concentration of 0.2% and 3% respectively. Before DNA extraction, an extra step was added to reduce the excess of polysaccharides, which in preliminary test were thought to inhibit DNA isolation and amplification. Therefore, 20-30 mg of plant material was ground and 1 ml of NaCl (5M) was added. Material was shaken (vibrational frequency 30 Hz, 28.00 agitations per second, 90 s) and then centrifuged at maximum revolution speed for 2 min in a standard tabletop centrifuge. The NaCl solution was then removed and the steps repeated 2-4 times.

DNA amplification of nuclear (ITS-internal transcribed spacer region of the 18S–5.8S–26S nuclear ribosomal cistron) and plastid regions (*matK- MaturaseK* gene*, trnT-L-trnT/trnL* spacer) were carried out by polymerase chain reaction (PCR). The ITS4 and NS5 standard primers were used to amplify the ITS region (Sun et al., 1994), and the *matK* intron and the *trnT-L* intergenic spacer were amplified using the 3914F and 1470R (Johnson and Soltis, 1994) and the a and b primers (Taberlet et al., 1991), respectively. Amplifications were unsuccessful in many samples (15 for ITS, 37 for *matK*, 31 for *trnT-L*) so we designed new internal primers for all regions based upon preliminary results, with two 21/20-nucleotide-long internal primers for ITS (ITS-intF: 5´-GTT GCG TGA CGC CCA GGC AG-3´; ITS-intR: 5´-GAG CAC AGC CTC CGT GGC TAG-3´); and two 21/20-nucleotide-long internal primers for *matK* region (matK-intF: 5´-GTC AAT TRA ATA AAT GGA TAG-3´; matK-intR: 5'-AGA GGA AGA CTC TTT TAM CC-3´). As expected, for the *trnT-L* region just one 21-nucleotide-long internal primer was amplified (trnTL-intF: 5´-GTA CAT ACG AAT TAC GCA AAC-3´), and this was combined with the standard primers a, b and d from Taberlet et al. (1991). DNA was amplified using a FlexCycler (AnalyticJena AG, Jena, Germany) or a 2720 ThermalCycler (AppliedBiosystems, Foster City, USA). After 4 min at 94ºC pretreatment, PCR conditions were set as follows: 39 cycles of 1 min at 94ºC, 1 min at 45ºC-58ºC, and 90 s at 72ºC. We added 0.2-0.8 µl of 10 mg/ml BSA (bovine serum albumin) in a total of 20 µl reaction volume in all reactions and 0.2-1 µl DMSO (dimethyl sulfoxide) was only included in reactions for ITS amplification. The PCR products were purified using spin filter columns (QIAquick PCR Purification Kit, California), following the manufacturer´s protocol. The cleaned product was then sequenced directly using dye terminators (*Big Dye Terminator v. 2.0, Applied*

Biosystems, LitteleChalfront, UK) following the manufacturer´s instructions and run in polyacrylamide electrophoresis gels (7%) using an Applied Biosystems Prism Model 3730 automated sequencer.

2.3 Phylogenetic analyses

To perform phylogenetic analyses, two matrices were constructed: one with 54 sequences (ingroup, 48; outgroup, 6) of the ITS region and the other one with 61 sequences (ingroup, 55; outgroup, 6) of the two concatenated plastid regions (*matK, trnT-L*). Sequences of ITS region, *matK* intron and *trnT-L* spacer, were aligned using MAFFT v.6.822 (Katoh, 2008) hosted on the CIPRES Science Gateway (Miller et al., 2010). The aligned sequences were inspected and corrected manually on BioEdit v.7.0.9.0 (Hall, 1999) to minimize the number of gaps following the method of Kelchner (2000). Phylogenetic analyses were performed using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) separately for nuclear (ITS) and for the concatenated plastid sequences (*matK, trnT-L*). The robustness for all nodes was estimated with posterior probability (PP) in BI and bootstrap values (BS) in both MP and ML.

For the Maximum Parsimony (MP) analysis, the dataset was analysed using equally weighted parsimony in TNT v.1.1 (Goloboff et al., 2008), with a heuristic search and a tree memory of 10000. Gaps were treated as missing data in all analyses. We chose 1,000 replicates of Wagner trees, followed by tree bisection-reconnection (TBR) branch swapping, and saved five trees per replication. A strict consensus tree was then generated. Nodal support was calculated using bootstrap resampling with 1,000 replications summarizing the absolute frequency of each group.

To adjust the BI and ML analyses with the most proximate model available, we tested the simplest model of sequence evolution that best fit the sequence data via the bottom-up strategy of hierarchical Likelihood Ratio Test (hLRT) and the Akaike Information Criterion (AIC, Akaike, 1979) using jModelTest v.0.1.1 (Guindon and Gascuel, 2003; Posada, 2008). The test was run separately for each of the three independent data sets (ITS, *matK, trnT-L*). The BI analysis was with MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003), and concatenated plastid sequences were treated as partitioned, implementing the GTR+Γ model. Data matrices were run for three million generations on four MCMC chains with a temperature of 0.2 with sampling every 100 generations. A consensus tree was calculated at a 50% majority-rule with the *sumt* command to obtain the final estimated phylogeny. The ML analysis was calculated using RaXML v.7.0.3

(randomized axelerated maximum likelihood for high performance computing; Stamatakis, 2006) with the GTRGAMMA mode and default settings for both nuclear (ITS) and plastid (*matK, trnT-L*) data sets separately, treating plastid sequence data set as partitioned. TenML searches were performed starting from 10 different randomized parsimony trees to obtain the best scoring tree. A standard nonparametric bootstrap with 100 replicates was carried out for internal support using the default estimation algorithm.

2.4 Divergence time estimates and DIVA analysis

Divergence time analysis for both nuclear and plastid data sets were performed under BI using BEAST 1.6.1 (Drummond and Rambaut, 2007). Xml-files for the BEAST analyses were constructed using BEAUti 1.6.1 (BEAST package). A combined matrix containing ITS, *matK* and *trnT-L* data sets was constructed to estimate the divergence times and concatenated datasets were analysed under partition-specific models. For the three genetic data sets we used the GTR+Γ model as the best fit substitution models, based on the AIC (Akaike, 1979). The data were analyzed under the uncorrelated lognormal relaxed clock model (UCLD) and a Yule Tree Prior was employed in all runs, which assumes a constant speciation rate for each branch of the tree.

The BEAST analysis was calibrated by using the same fossil records as described in Guzmán and Vargas (2009a). The tree root, consisting of the divergence time of Dipterocarpaceae and Cistaceae, was constrained with a minimum of 23 Ma and a maximum of 39 Ma, following Wikström et al. (2001). The prior for the age of the root was therefore set to a normal distribution with a mean of 31 Ma and a standard deviation of 4.1 Ma. We chose a normal distribution as it places higher probability on intermediate dates, providing a more appropriate prior calibration (Ho and Phillips, 2009).

The crown group of the Cistaceae family was constrained using the earliest fossil found of the family of the Cistaceae. This fossil was described as a reproductive structure of *Cistinocarpum roemeri* Conis (Palibin, 1909) and dated from the Middle Oligocene in Germany (28 Ma old). We therefore employed a lognormal distribution prior with an offset of 28 Ma and a standard deviation of 0.8 Ma

The MCMC post chain was run with for 50 x 10^6 generations (with a burnin of approximately 10%) and sampled every 1000th generation. Tracer v.1.4 (Rambaut and Drummond, 2007) was then used to measure the effective sample size (ESS) of each parameter, which in all cases exceeded 100. Trees were then summarized with Tree Annotator v.1.6.1 (Rambaut and Drummond, 2010) as maximum clade credibility, mean node heights and a 0.5 posteriori probability limit. FigTree v.1.3.1 (Rambaut, 2009) was used to visualize the tree.

To reconstruct ancestral areas of distribution, a dispersal-vicariance analysis (S-DIVA) was performed using RASP v.2.0 beta (Yu et al., 2010). This method resolves the phylogenic uncertainty of using a collection of trees. DIVA allows the reconstruction of ancestral distributions without any previous assumptions about the area (Ronquist, 1997), and its use has been recommended under reticulated biogeographical scenarios, such as the Mediterranean Basin (Sanmartín, 2003; Oberprieler, 2005). After discarding 10,000 trees from a BI analysis of the nuclear (ITS) and plastid (*matK*, *trnT-L*) data, we employed a subsample of 20,000 trees with the slow ancestral reconstruction option selected to infer ancestral distribution areas. To define the areas, a paleographical criterion was followed (Meulenkamp and Sissingh, 2003) and the selected areas were: A, northwestern Mediterranean; B, southwestern Mediterranean; C, southeastern Mediterranean; D, northeastern Mediterranean; E, Eurosiberian (Table 1; Fig. 3). The biogeographical analysis was restricted to a maximum number of five areas, given that this is the maximum number of areas occupied by *Fumana procumbens* Gren. &Godr., the more widespread species. Outgroups from the Dipterocarpaceae were excluded from the analysis and were coded as "null", according to Yu et al. (2012).

2.5 Ancestral state reconstruction

There are 15 morphological characters that have traditionally been considered for circumscription of the genus *Fumana* (Güemes, 1991).For the analysis of character evolution we chose six characters which are considered taxonomically important for the genus *Fumana* and one (mucilage secretion in seeds) which has not been studied before. Therefore, a total of seven characters of seed morphology (dispersal unit; seed vs. fruit), number, mucilage secretion and ornamentation), leaf morphology (margin and form) and trichome types (presence of glandular trichomes) were analyzed and mapped on a pruned total evidence phylogeny. Character states were determined for each species from fresh and herbarium material. The complete morphological matrix was performed coding for a total of seven characters. We used the “drop.tip” command of the “ape” software (Paradis et al., 2013) in R v.3.0.1 (R Core Team, 2013) to prune the tree, excluding repeated species. To infer patterns of character evolution, we used the ML function of Mesquite v.2.74 (Maddison and Maddison, 2009) to trace character states on the consensus tree obtained from the BI analysis. The “Trace Character History” option was used under the likelihood reconstruction method to display the ancestral state. The maximum likelihood model provides information on genetic branch lengths and uses the Markov *k*-state one parameter model (Mk1), which assumes a single rate for all transitions between character states (Lewis, 2001). Character states with a significant likelihood for reconstruction were considered the most likely ancestral states (i.e., using the average likelihood decision threshold of 2.0, with a proportional likelihood of 0.88 or higher(Maddison and Maddison, 2009).

3 Results

3.1 Phylogenetic analyses

In the genus *Fumana*, *trnT-L* sequence diversity, using the K-2-p model of evolution, ranged from 0.0% (between 31 conspecific accessions and between *F. ericifolia* - *F. paradoxa*, *F. juniperina* - *F. thymifolia*) to 5.1% (between *F. laevipes* - *F. trisperma*); *matK* sequence diversity ranged from 0.0% (in 36 conspecific accessions and 11 congeneric accessions) to 3.3% (between *F. arabica* and *F. laevipes*); and ITS sequence diversity varied from 0.0% (between 14 conspecific accessions and 11 congeneric accessions) to 3.1% (between *F. arabica* and *F. thymifolia*, Table 3). The genus *Fumana* was recognized as monophyletic in the BI, MP, and ML analyses using ITS (100 PP, 100% BS, 100% BS, respectively, Fig. 2) and *matK* and *trnT-L* sequences (100 PP, 98% BS, 92% BS, respectively, Fig. 1).

The BI, MP and ML analyses of *matK* and *trnT-L* sequences yielded similar topology with BI displaying higher values (Fig. 1). The ML and MP analysis had lower resolution and lower support, only adding a well-supported clade with two conspecific accessions of *Fumana thymifolia* in ML (accessions number 2 and 3). When the GTR+G was used as the simplest model, the BI analysis for the combined matrix of plastid sequences reached equilibrium after 75,000 generations. Six conspecific accessions formed well supported monophyletic groups in BI, MP and ML (with exception of *F. ericoides* with a 62% BS in MP and *F. baetica* with 63% BS in MP and 70% BS in ML). The consensus tree of the BI, MP and ML analysis of plastid regions revealed four major clades (named I, II, III, IV), where clades I, II and IV were strongly supported with BS values ≥85% and Bayesian PP >0.95. Clade I was clustered together with clade II with low PP and BS support (78 PP in BI, <50% BS in MP, 74% BS in ML); while clade III clustered with clade IV with high PP and moderate BS values (97 PP in BI, 77% BS in MP, 70% BS in ML).

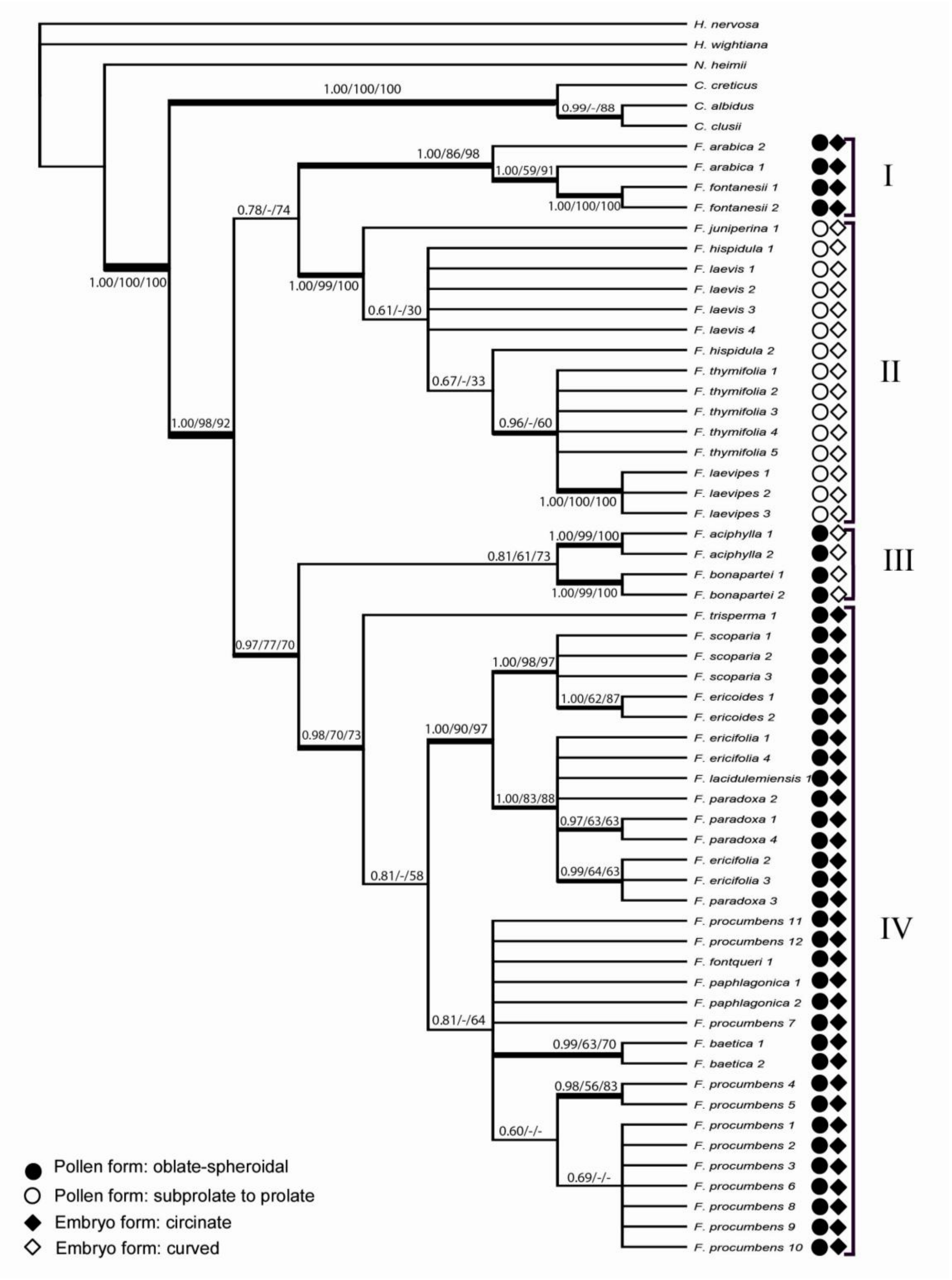

Figure 1: Majority consensus tree (50%) from BI analysis based on plastid sequences (*trnT-L*, *matK*) of *Fumana* species. Population numbers are given after species name (see Table 2). Above branches: BI Posterior Probability/MP Bootstrap/ML Bootstrap. Strict consensus tree of 475 MP trees (CI = 0.805, RI = 0.941, 206/1059 steps).A hyphen represents incongruence between BI tree and MP or ML consensus tree. Branches with posterior probability ≥0.95 are represented by thick lines.

Two of the four major clades partially follow the traditional separation in two subgenera, with clades II and IV containing almost all species previously grouped in the *Fumanopsis* and *Fumana* subgenera respectively (6 out of 8 in *Fumanopsis,* and 10 out of 12 in *Fumana*). Clade II includes five species of which two (*Fumana thymifolia* and *F. laevipes*) clustered together with 96 PP in BI, 60% BS in ML and <50% BS in MP. Clade IV clusters 10 species formed by *F. trisperma* and by two other groups of species. One group clusters four species (*F. procumbens*, *F. fontqueri*, *F. paphlagonica* and *F. baetica*) and the other holds five species (*F. scoparia*, *F. ericoides*, *F. ericifolia*, *F. lacidulemiensis* and *F. paradoxa*). *F. scoparia* and *F. ericoides* clustered together (100 PP in BI, 98% BS in MP, 97% BS in ML) and were separated from the monophyletic clade of *F. ericifolia*, *F. paradoxa* and *F. lacidulemiensis* (100 PP in BI, 83% BS in MP, 88% BS in ML).

A majority rule consensus tree of the nuclear ITS sequences from BI under the GTR+G model is shown in Fig. 2, with the corresponding values of ML and MP analyses. The BI analysis reached equilibrium after 100,000 generations. ITS sequence data produced limited resolution with unresolved polytomies in all three analyses. Monophyly for *Fumana* was only clearly supported by in the MP analysis (100% BS), while BI and ML analyses depicted *Fumana* with *Cistus* as a monophyletic clade (100 PP in BI, 100% BS in ML). The ITS phylogeny revealed monophyly in six species, with 3 well supported groups of conspecific accessions in the BI, MP and ML analyses (*Fumana fontanesii*, *F. laevipes* and *F. thymifolia*). There was a partial congruence in the topology to the plastid tree, since accessions of clades II and III in the *matK* and *trnT-L* analyses were also clustered together (clade II: 100 PP in BI, 99% BS in MP and 98% BS in ML; clade III: 97 PP in BI, <50% in MP, 53% in ML) in the ITS analysis.

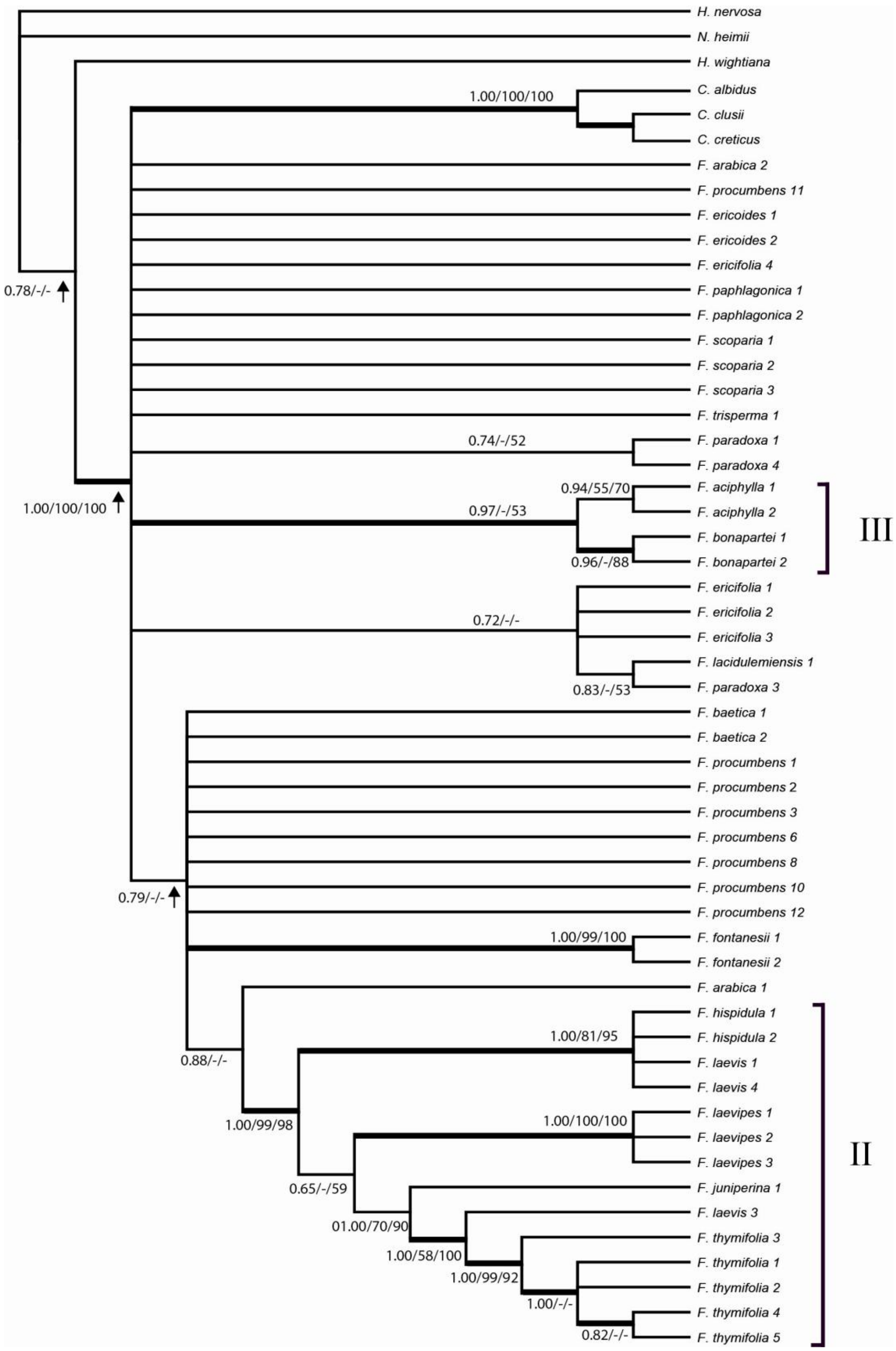

Figure 2: Majority consensus tree (50%) from BI analysis based on nuclear sequences (ITS) of Fumana species. Population numbers are given after species name (see Table 2). Above branches: BI Posterior Probability/MP Bootstrap/ML Bootstrap. Strict consensus tree of 400 MP trees (CI = 0.704, RI = 0.884,

176/813 steps). A hyphen represents incongruence between BI tree and MP or ML consensus tree. Branches with posterior probability ≥0.95 are represented by thick lines.

3.2 Divergence time estimates and DIVA analysis

According to our molecular dating analysis, divergence times of *Fumana* took place about 16.97 Ma ago (24-10 Ma). The first branch to separate was the group formed by clade I (*Fumana arabica* and *F. fontanesii*) and clade II (*Fumanopsis*) in the Miocene about 14.6 Ma ago (21-8.8 Ma). The second branch to separate was the group that included clades III and IV, located in the Miocene about 11.8 Ma (17.5-6.3 Ma).

Table 1 and Fig. 3 show the results of the dispersal-vicariance analysis. The analysis established the area for the ancestor of the genus in the northwestern Mediterranean area with a probability of 84.55%. Results support that species divergence in nodes c, e, f and g occurred through a dispersal event with a probability higher than 0.80. One vicariance event was also detected in node d (probability >0.8756).

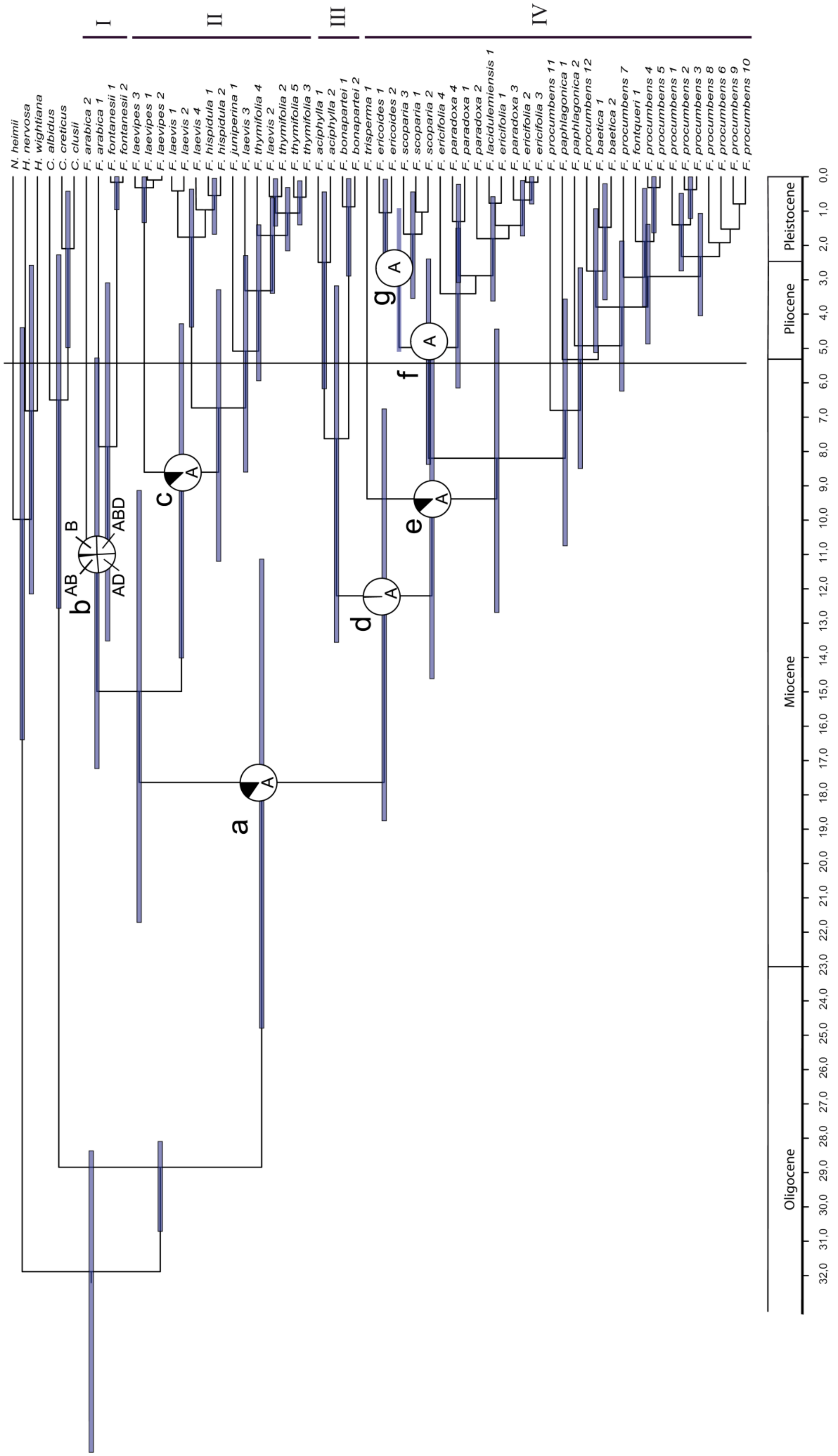
I
II
III
IV
N. heimii
H. nervosa
H. wightiana
C. albidus
C. creticus
C. clusii
F. arabica 2
F. arabica 1
F. fontanesii 1
F. fontanesii 2
F. laevipes 3
F. laevipes 1
F. laevipes 2
F. laevis 1
F. laevis 2
F. laevis 4
F. hispidula 1
F. hispidula 2
F. juniperina 1
F. laevis 3
F. thymifolia 4
F. laevis 2
F. thymifolia 2
F. thymifolia 5
F. thymifolia 3
F. aciphylla 1
F. aciphylla 2
F. bonapartei 1
F. bonapartei 2
F. trisperma 1
F. ericoides 1
F. ericoides 2
F. scoparia 3
F. scoparia 1
F. scoparia 2
F. ericifolia 4
F. paradoxa 4
F. paradoxa 1
F. paradoxa 2
F. lacidulemiensis 1
F. ericifolia 1
F. paradoxa 3
F. ericifolia 2
F. ericifolia 3
F. procumbens 11
F. paphlagonica 1
F. paphlagonica 2
F. procumbens 12
F. baetica 1
F. baetica 2
F. procumbens 7
F. fontqueri 1
F. procumbens 4
F. procumbens 5
F. procumbens 1
F. procumbens 2
F. procumbens 3
F. procumbens 8
F. procumbens 6
F. procumbens 9
F. procumbens 10
a
A
b
AB
B
ABD
AD
c
A
d
A
e
A
f
A
g
A
0,0
1,0
2,0
3,0
4,0
5,0
6,0
7,0
8,0
9,0
10,0
11,0
12,0
13,0
14,0
15,0
16,0
17,0
18,0
19,0
20,0
21,0
22,0
23,0
24,0
25,0
26,0
27,0
28,0
29,0
30,0
31,0
32,0
Pleistocene
Pliocene
Miocene
Oligocene

Figure 3: Chronogram obtained with BI dating of the combined data (ITS, *trnT-L*, *matK*). The pie charts represent the relative probability of ancestral areas reconstructed for each node using the DIVA analysis. The black portion represents reconstructions with a probability <0.10. Letters next to pie charts and terminals correspond to areas (A, north-western Mediterranean; B, south-western Mediterranean; C south-eastern Mediterranean; D, north-eastern Mediterranean). Ancestral area reconstructions are only indicated for nodes with phylogenetic support (PP ≥0.90; ≥70%).

Table 1. Results of the DIVA analysis. Nodes refer to Fig. 3. (A, north-western Mediterranean; B, south-western Mediterranean; C south-eastern Mediterranean; D, north-eastern Mediterranean).

Node	Height (Ma)	Height 95% (Ma)	Event	Reconstruction	Probability
a	18.29	24.92-11.85	Dispersal	A → A/ABD	0.2322
b	11.34	17.83-5.49	Dispersal	B → AB/DB	0.2463
c	9.41	14.53-4.55	Dispersal	A → ABCD/A	0.8015
d	13.10	19.49-7.42	Vicariance	AD→ A/D	0.8756
e	10.08	15.08-5.5	Dispersal	A→AD/A	0.2863
f	5.51	11.29-3.88	Dispersal	A→A/A	1
g	2.88	5.09-0.91	Dispersal	A→ABCD/A	1

3.3 Ancestral state reconstruction

Ancestral states of all seven characters were reconstructed for all nodes of the tree and are shown in Figs. 4a-d. The character state reconstruction showed that seed number and ornamentation were equivocally reconstructed. We found a higher likelihood for a nine-seeded state as an ancestral reconstruction, but it was not statistically significant (Fig. 4a). Clades II and IV separated, respectively, species with six reticulated seeds and species with nine papillated seeds. However, three-seeded *Fumana aciphylla* and *F. trisperma* are separated into clades I and IV while six-seeded *F. bonapartei* is located in clade III. The two types of seed ornamentation are present in both clades I and III.

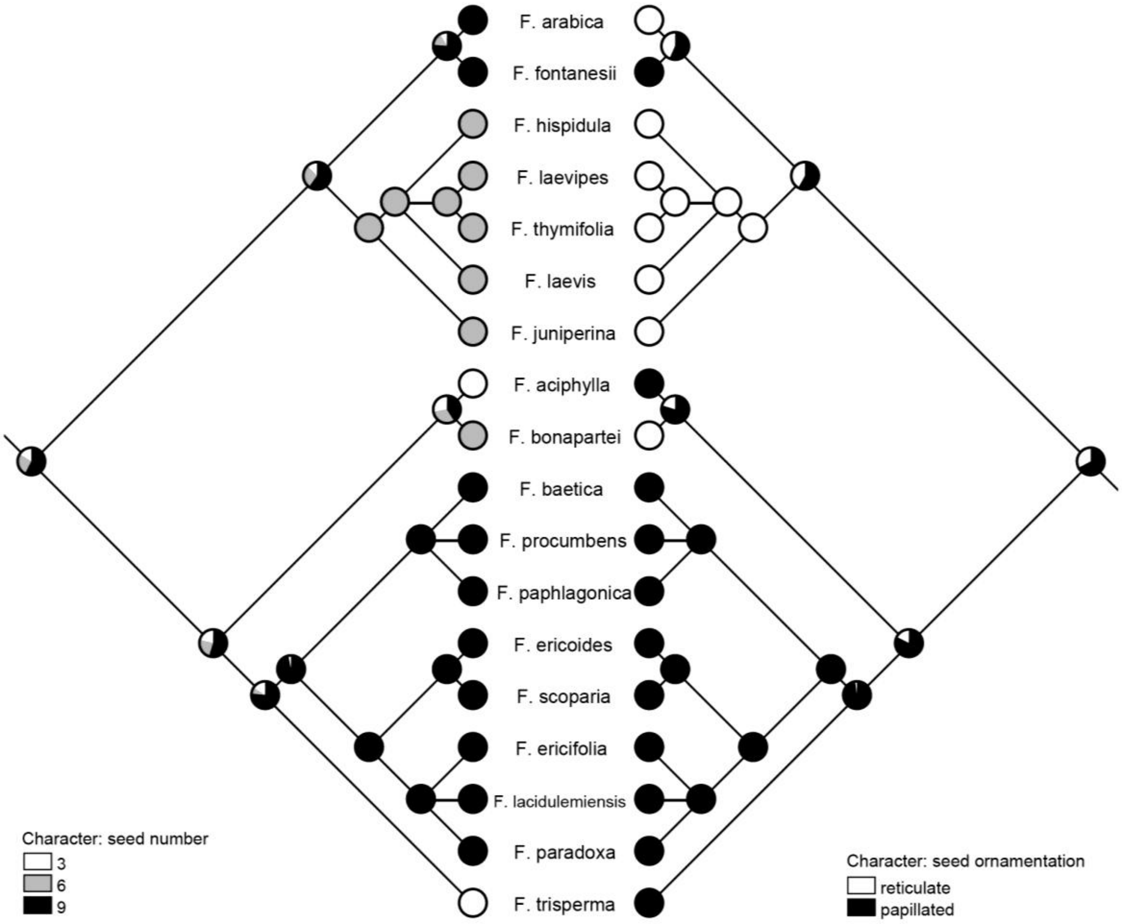

Figure 4a: Likelihood-based ancestral state reconstruction of seven selected morphological-anatomical characters. Proportional likelihoods of the most likely state are shown at nodes for all species and clades. **Seed number and seed ornamentation** characteristics are mapped onto the majority consensus tree (50%) from BI analysis based on plastid sequences (*matK* and *trnT-L*).

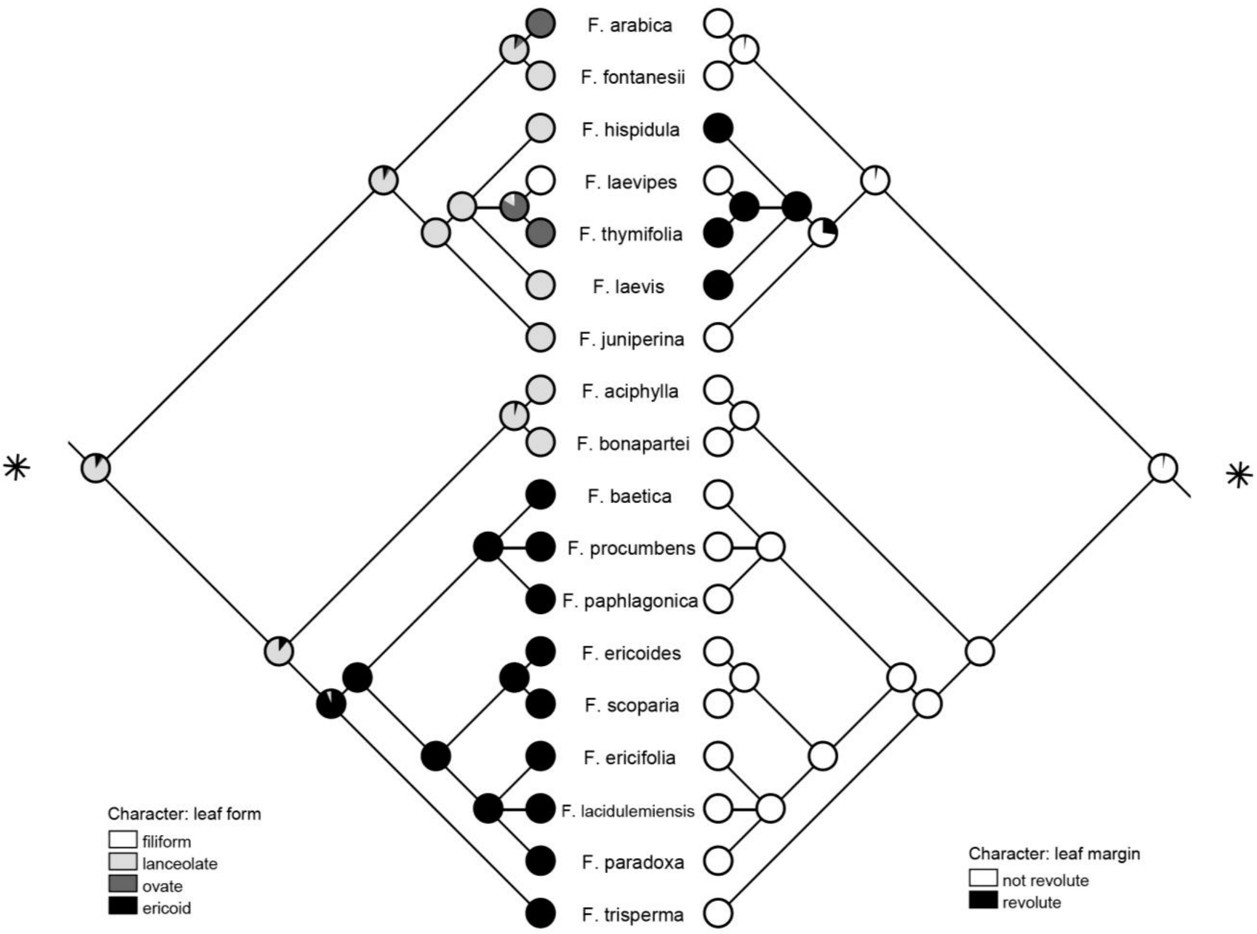

Figure 4b: Likelihood-based ancestral state reconstruction of seven selected morphological-anatomical characters. Proportional likelihoods of the most likely state are shown at nodes for all species and clades. **Leaf margin and leaf form** are mapped onto the majority consensus tree (50%) from BI analysis based on plastid sequences (*matK* and *trnT-L*).

Character optimization was significant on reconstructing the leaf margin and leaf form (Fig. 4b). No revolute leaf margin was reconstructed as the most likely ancestral state with a change to revolute leaf margin in three species of clade II. Lanceolate leaf form was the most likely ancestral state, which changed to ericoid in clade IV, and to ovate in *Fumana arabica* and *F. thymifolia*, in clade II. *F. laevipes* was the only species shifting to a filiform shape. Glandular trichomes were reconstructed as ancestral state and have changed twice to non-glandular trichomes, once in clade IV and *F. fontanesii* in clade I (Fig. 4d).

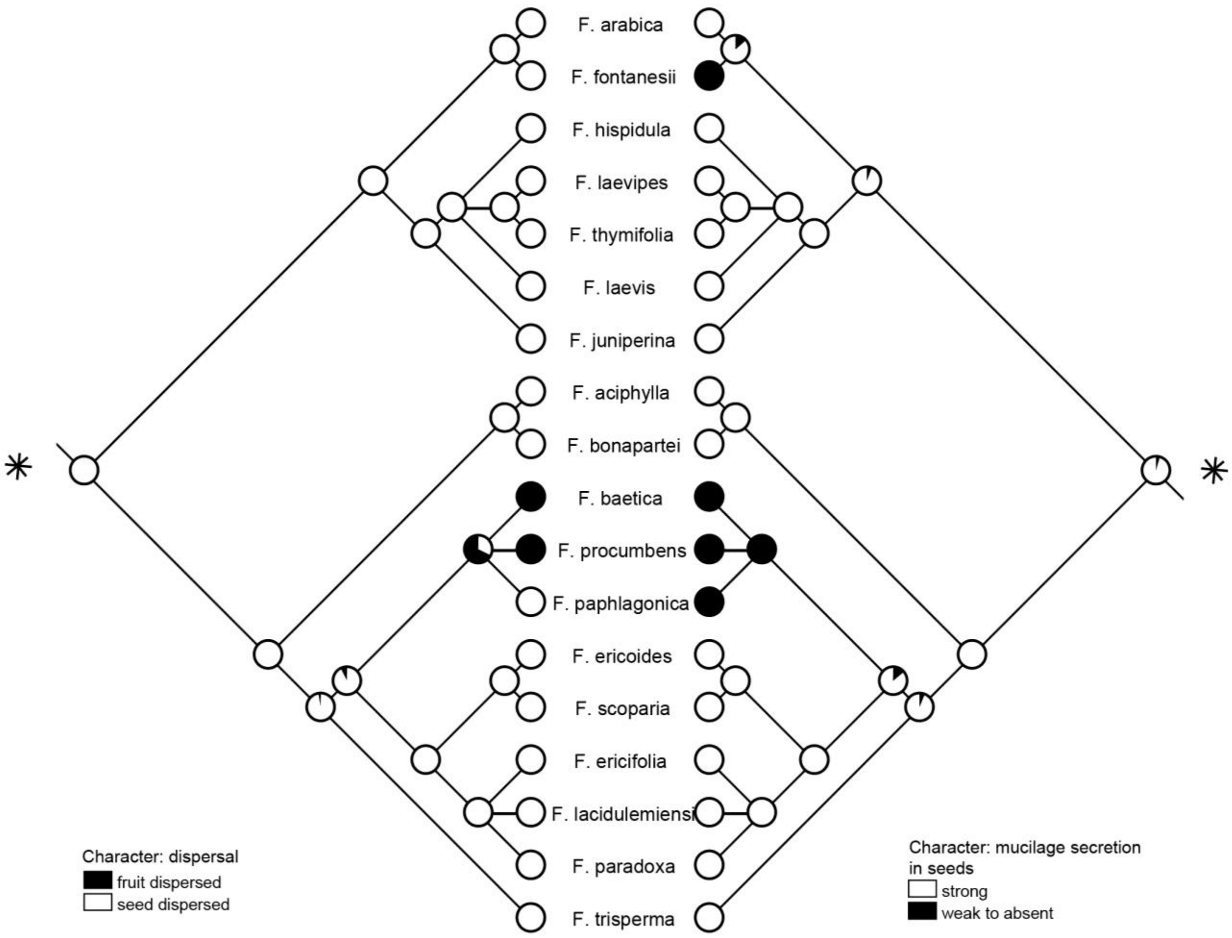

Figure 4c: Likelihood-based ancestral state reconstruction of seven selected morphological-anatomical characters. Proportional likelihoods of the most likely state are shown at nodes for all species and clades. **Dispersal and mucilage secretion** are mapped onto the majority consensus tree (50%) from BI analysis based on plastid sequences (*matK* and *trnT-L*).

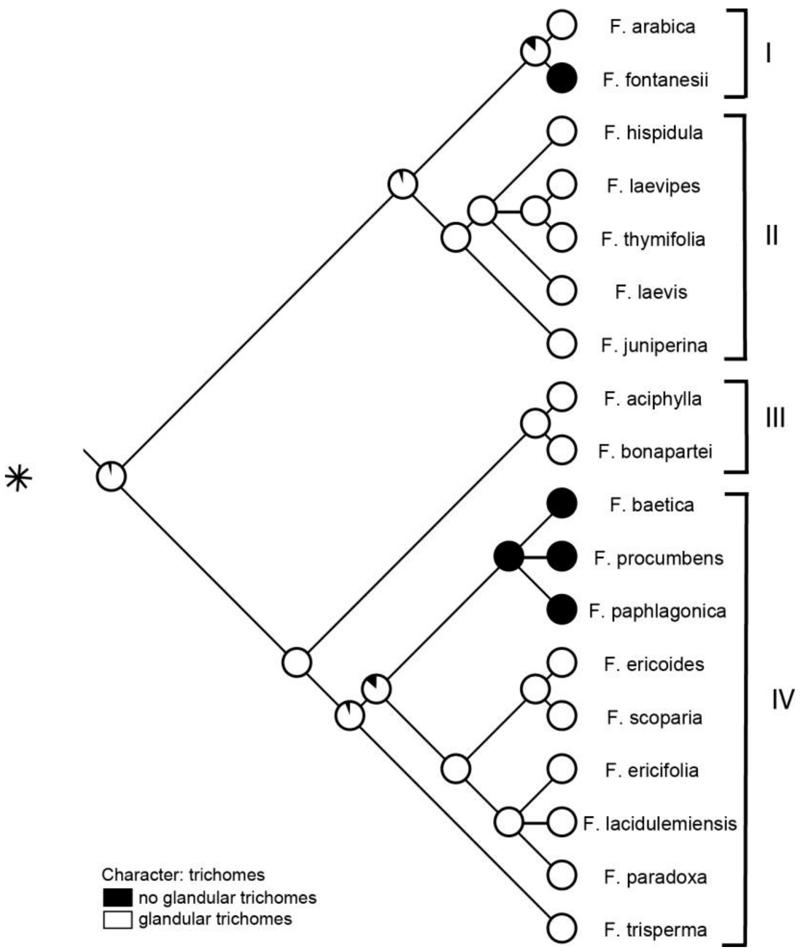

Figure 4d: Likelihood-based ancestral state reconstruction of seven selected morphological-anatomical characters. Proportional likelihoods of the most likely state are shown at nodes for all species and clades. **Presence of glandular trichomes** is mapped onto the majority consensus tree (50%) from BI analysis based on plastid sequences (*matK* and *trnT-L*).

The diaspore only changed in two species in clade IV from seed to fruit dispersed (Fig. 4c). Seed dispersal has been reconstructed as a significant ancestral state while fruit dispersal is shown to be a derived character. Strong mucilage secretion in seeds of *Fumana* has been significantly reconstructed as an ancestral state and is present throughout most of the clades. There were two changes in a very weak or absent mucilage secretion of seeds in clades IV and I, in the latter, only in *F. fontanesii*.

4 Discussion

4.1 Phylogenetic analyses and systematic implications

Analysis of the combined plastid sequences of *Fumana* significantly supports the monophyly (100PP, 100% BS in MP and 92% BS in ML) of the genus in Cistaceae, according to Guzmán and Vargas (2005; 2009a). However, these results do not support the traditionally established infrageneric divisions of this genus (supplementary Table 1). Former authors tried to categorize the high morphological diversity and proposed divisions of the genus (Willkomm, 1856; 1880). With extended knowledge about of genus *Fumana* and the successive description of new species throughout the 20th Century, some species did not fit into any of the previously established groups, and these infrageneric divisions were successively revised. Janchen (1920) established the most complex division by separating the genus in two subgenera based on embryologic characters, and then each one into two sections based on vegetative characters. In some occasion, the genus was divided into two (Pomel, 1860) or three genera (Raynaud, 1992), although this approach was never approved by other botanists.

Our results confirm the presence of four clades of species (Fig. 1), in which the two main clades (II and IV) correspond with the proposal of Ukraintseva (1993), based on the characteristics of the pollen grain and the proposal of Janchen (1920) based on the characteristics of the embryo form (Fig. 1). Clade II primarily clusters species originally placed in the subgenus *Fumanopsis*, bearing subprolate to prolate pollen with a granular-reticulate ornamentation and a curved embryo. Clade I comprises species formerly placed in the classic subgenus *Fumana,* with different pollen and seed characteristics to clade II, as well as different vegetative and reproductive characters. Clades III and IV are composed of species traditionally placed in the subgenera *Fumanopsis* and *Fumana*, respectively, with oblate-spheroidal shaped pollen with retipilate ornamentation. However, these clades differ in their embryologic characters. Clade III clusters species with three to six homomorphous seeds with a curved embryo, while clade IV clusters species with nine heteromorphous seeds with a circinate embryo (Fig. 1 and Fig. 4a).

We find a concordance of 80% (15 of 19 studied species) between the molecular phylogenetic and the classic infrageneric divisions in clades II and IV. However, clades I and III are not in accordance with the established taxonomic divisions. Both clades hold two species that are morphologically the most inconsistent and conflicting ones of the whole genus. The two species in clade I and III share very few characters with the adjacent clade and do not share common characters that clearly define them as a group. Therefore, even though the molecular analysis confirms the presence of four clades, species in the clades defined by the molecular approach do not share enough vegetative or reproductive characters to clearly delimit them as a subgenus. Consequently, and in concordance with Grosser (1903), these findings neglect any infragenetic division and therefore we suggest invalidating the independency of the three subgenera.

Focusing on the details of the taxonomic analysis, our study supports the separation of some of the species frequently discussed in recent years. This is the case of *Fumana baetica*, a species commonly confounded with *F. procumbens* and *F. paradoxa*(Güemes, 1989), which shows a well-defined monophyletic group in our phylogenetic analysis, separated from the other two taxa. *Fumana ericoides* and *F. scoparia* also clustered as two independent species, as previously proposed by Cavanilles (1793) and Coode and Davis (1964). It now appears they are clearly separated from the other species in the group, like *F. ericifolia* and *F. paradoxa*, as maintained in the most recent reviews of the genus(Güemes and Molero, 1993). The group of *F. thymifolia* also forms a clade apart, confirming the separation from the group of species having glabrous and opposite positioned leafs (*F. juniperina*, *F. hispidula*, *F. laevis*). *Fumana fontanesii*, *F. laevipes*, *F. aciphylla*, *F. bonapartei* and *F. trisperma*, whose morphological characters strongly differ from each other and from the rest of species in the genus, which had always been considered by taxonomists as independents, are confirmed as monophyletic groups in our phylogeny.

4.2 Divergence time estimates and DIVA analysis

Based on the analysis of dating, the origin of the genus is between the Early and the Middle Miocene (Fig. 3, Table 1), while the divergence of major clades occurred between 15.61 and 8.8 Ma, between the Middle and Late Miocene, followed by an important process of diversification during the Pliocene (5.3-2.6 Ma). Our results agree with those obtained in other Mediterranean genera, with different life forms and biogeographic histories, such as *Cistus* (Guzmán and Vargas, 2005), *Antirrhinum* (Vargas, 2009), *Dianthus* (Valente et al., 2010), *Narcissus* (Santos-Gally et al., 2012) or *Erodium* (Fiz-Palacios et al., 2010), whose diversification also occurred mainly between the late Late Miocene and Pleistocene.

During the Cenozoic, there was an increase in aridity that ended in the Messinian Mediterranean salinity crisis (5.96-5.33 Ma, Bocquet et al., 1978) and the subsequent stabilization of the Mediterranean climate 2.8 Ma ago (Suc, 1984). These changes had a great impact in the Mediterranean Basin, and suggest a strong relationship between the subsequent period of climatic stability and the high diversity of species in several groups of Mediterranean plants during this time (Fiz-Palacios et al., 2010; Fernández-Mazuecos and Vargas, 2011). Within *Fumana*, the major species diversity occurred in the Late Pliocene and could be interpreted as a sign of ancestral divergence associated with adaptation to the onset of seasonal drought, characteristic of the Mediterranean climate. Many species of the genus (e.g. *F. ericoides* or *F. thymifolia*) are well adapted to fire and drought and are effective colonizers of disturbed environments (Arnan et al., 2010; Moreira and Pausas, 2012) and also probably due to their selfing ability (Güemes and Boscaiu, 2002; Carrió et al., 2008; Carrió and Güemes, 2013).

The results of the reconstruction of areas suggest a western Mediterranean ancestor for the genus *Fumana* (Fig. 3, Table 1). Based on the number of species, historical biogeography highlighted the western Mediterranean as the main center of diversification, and the eastern Mediterranean as a secondary center for this genus. Our analysis also suggests that at least two colonization processes in the Late Miocene and Pliocene (9.41 Ma and 2.88 Ma) from the NW to the NE, and from SW to SE Mediterranean; and one vicariant process between NW and NE lineages in Medium Miocene. These events would explain the origin of the secondary diversification centre for *Fumana* in the eastern Mediterranean and coincide with other proposals to explain the current species distribution of other Mediterranean plant groups (Galicia-Herbada, 2006; Font et al., 2009).

4.3 Ancestral state reconstruction

Results of character reconstruction suggested that shifts in seed, leaf and trichome characters occurred in the diverse clades of *Fumana* (Fig. 4a-d).

The leaves of *Fumana* have strong sclerophyllous characters with a thick cuticle covered by dense hair (Güemes, 1991). Leaf modifications are important adaptations to arid environments (Puigdefábregas and Pugnaire, 1999). By reducing their evaporation surface, plants may reduce water loss and therefore, small leaf size, especially narrow ones, are generally favoured under high sun exposure and low water availability (Parkhurst and Loucks, 1972; Givnish and Vermeji, 1976; Witkowski and Lamont, 1991). In our character reconstruction the ancestral state appears to be a strongly reduced leaf area (lanceolate) which was maintained in three clades, but evolved to a still stronger reduction (filiform in *F. laevipes*), and to an ericoid leaf form only in clade IV. Furthermore we found one transition to revolute leaf margin in clade II, reducing the evaporation area even more. Still, *F. juniperina* and *F. laevipes* do not show revolute leaf margins but their leaves have a strongly reduced surface where the margins simply cannot rollup any further. The leaf form is not the only trait related to arid conditions. Trichomes have been described as characters related to both water control and resistance against herbivores in various plant species (Levin, 1973; Ehleringer et al., 1976; Woodman and Fernandes, 1991). Under strong drought conditions and in highly insulated environments, trichomes decrease the head load over the surface area and also radiation absorbance, thus reducing evaporation and water loss (Wuenscher, 1970; Ehleringer et al., 1976). In *Fumana* leaves, two main types of trichomes (glandular and non-glandular) have been found, and then being simple or pluricellular hair. In the genus *Fumana,* we found that glandular trichomes are the ancestral state, and are maintained in most species, indicating the adaptation to arid and drought conditions under strong radiation, as water loss due to evaporation can be reduced. Meanwhile the loss of glandular trichomes occurs in only four species and is linked to species with a broader leaf form inhabiting moister environments (clade IV, Fig. 4d).

Furthermore, our data suggests that nine seeded capsules are the ancestral state. The evolution of different levels of allocation to reproduction is thought to be driven largely by the level of disturbance in the habitat (Fenner and Thompson, 2005). Ovaries of *Fumana* have four ovules in three carpels, in which one of each is aborted to reach a maximum of nine seeds per capsule (Güemes, 1991). Water stress during flowering has been shown to almost totally inhibit or prevent fruit development in some species (Gusta et al., 1997; Aragón et al., 2008; Whittle et al., 2009) by abortion or reduced megagametophyte fertility (Young et al., 2004). Regulating reproduction and resource allocation during drought stress could improve plant resources and benefit persistence in a changing environment, as hypothesized by Aragón et al. (2008) and Sun et al. (2004). These adaptations usually act over a short time period, but could have initiated the reduction from 12 seeds (currently non-existent) to nine, six or three seeds per capsule, characteristic of the different species of *Fumana*, due to environmental changes.

Seeds of many *Fumana* species produce a sticky and thick mucilaginous layer around the seed coat when they come into contact with water. The gel is made of polysaccharides and adheres them to the soil upon drying (Grubert, 1974; Werker, 1997; Engelbrecht et al., 2013). Our results show that strong mucilage secretion is an ancestral state in *Fumana* but also that it changed twice into a weak or absent state of mucilage, particularly in clades I and IV. The dispersal unit in *Fumana* is predominantly the seed. However, a shift to fruit-dispersal appeared in three species in clade IV. We hypothesize that the simultaneous absence of mucilage and the dispersal of seeds inside fruit (fruit dispersed) could be related to high mountain habitats in the Mediterranean, as it is the habitat of the species *F. baetica*, *F. procumbens* and *F. paphlagonica* of clade IV. Mucilage secretion in seeds in these habitats could place the seed in danger of getting glued onto rocks and stones were seedling growth is impossible. In contrast, dispersal of seeds inside the fruit may increase the possibility of fruits getting trapped inside their own or other plant structures or in rocky fissures, a mechanism that also enhances survival, as seeds can fall out of the fruit once it opens on the ground. An in-depth study should be conducted to confirm this hypothesis.

Table 2: List of studied material with accession number in brackets after species name, species distribution (A, north-western Mediterranean; B, south-western Mediterranean; D, north-eastern Mediterranean; C south-eastern Mediterranean; E, Eurosiberian), locality, geography coordinate, voucher sample and ITS, *matK*, *trnT-L* GenBank accession numbers.

Taxon	**Distribution**	**Locality**	**Geographic coordinate**	**Voucher sample**	**Accesion no. ITS**	**Accesion no. *matK***	**Accesion no. *trnT-trnL***
Fumana (Dunal) Spach							
F. aciphylla Boiss. (1)	D	Greek: Grevena, Varis	40º8’N/21º37’W	FJC778, VAL188194	KJ534144	KJ534086	KJ534192
F. aciphylla Boiss. (2)		Turkey: Erzincan Koçyatagi, Sakaltutan Geçidi	39º52’N/39º7’W	JA2697, VAL146325	KJ534145	KJ534087	KJ534193
F. arabica Spach (1)	DB	Cyprus: Famagusta, Karpasian Peninsula	35º16’N/33º53’W	JRV5630, VAL189027	KJ534146	KJ534088	KJ534194
F. arabica Spach (2)		Greek: Kalavryta, Mega Spileon monastery	37°58’N/22°18’W	JC0811, VAL190953	KJ534147	KJ534089	KJ534195
F. baetica Güemes (1)	A	Spain: Jaen, Cazorla, Sierra de Cazorla, Guadahornillos	37º55’N/2º50’W	JG s/n, VAL26605	KJ534148	KJ534090	KJ534196
F. baetica Güemes (2)		Spain: Cuenca, Las Catedrales	40º14’N/01º58’W	JG4230, VAL207028	KJ534149	KJ534091	KJ534197

F. bonapartei Maire & Petitm. (1)	D	Greek: Kozani Palaiokastro, Mt. Vourinos	40º11’N/21º38’W	FJC808, VAL190472	KJ534152	KJ534094	KJ534200
F. bonapartei Maire & Petitm. (2)		Greek: Ioannina Pades, Mt. Smolikas, Dracolimni	40º4’N/20º54’W	RG827, VAL191581	KJ534153	KJ534095	KJ534201
F. ericifolia Wallr. (1)	ABCD	Spain: Cádiz, Grazalema, Puerto de las Palomas	36º47’N/5º22’W	JG4192, VAL189048	KJ534159	KJ534101	KJ534207
F. ericifolia Wallr. (2)		Spain: Murcia, Benizar	38º16’N/1º59’W	JG4073, VAL181308	KJ534160	KJ534102	KJ534208
F. ericifolia Wallr. (3)		Spain: Cuenca, Alarcón	39°32’N/2°5’W	JGs/n-1 BdB 566	KJ534157	KJ534099	KJ534205
F. ericifolia Wallr. (4)		Tunisia: Kasserine Governorate, Dashrat Zawiyat Sidi Salis, Djebel Chambi	35º12’N/8º40’W	JC3239, VAL201881	KJ534158	KJ534100	KJ534206
F. ericoides Wallr. (1)	A	Spain: Alicante, La Nucía, San Vicente chapel	38º36’N/0º6’W	JGs/n-2 BdB 10	KJ534155	KJ534097	KJ534203
F. ericoides Wallr. (2)		Spain: Valencia, Serra, Porta-Coeli	39º39’N/0º28’W	ECA161, VAL203638	KJ534156	KJ534098	KJ534204
F. fontanesii Clauson ex Pomel (1)	AB	Morocco: Taza-Al Hoceima-Taounate, Aknoul	34º38’N/3º51’W	ABH55407	KJ534162	KJ534104	KJ534210
F. fontanesii Clauson ex Pomel (2)		Spain: cultivated in the Botanical Garden of the University of Valencia from seeds collected in Alhama de	39º28’N/0º23’W	ECA250, VAL 207011	KJ534161	KJ534103	KJ534209

		Murcia					
F. fontqueri Güemes (1)		Morocco: Tanger-Teouan, Bab Taza, Parc National Talassemtane, Jbel Lakraa	35º8’N/5º8’W	AQ2774, MA782664	-	KJ534105	KJ534211
F. hispidula Loscos & Pardo (1)	A	Spain: Valencia, El Saler, Les Gavines	39º21’N/0º19’W	ECA81, VAL189070	KJ534163	KJ534106	KJ534212
F. hispidula Loscos & Pardo (2)		Spain: Teruel, Castelserás	40°58’N/0°8'W	JG4239, VAL207031	KJ534164	KJ534107	KJ534213
F. juniperina (Lax. ex Dunal) Pau (1)	ABC	Tunisia: Bizerte Governorate, Sidi Ferdjani, Cap Serrat	37º13’N/9º13’W	AQ3028, VAL201342	KJ534165	KJ534108	KJ534214
F. lacidulemiensis Güemes (1)	A	Spain: Cádiz, Grazalema, Puerto de las Palomas	36º47’N/5º22’W	JG4190, VAL189059	KJ534167	KJ534110	KJ534216
F. laevipes Spach (1)	ABCD	Tunisia: Nabeul Governorate, Korbous	36º49’N/10º34’W	AQ3303, VAL201343	KJ534171	KJ534115	KJ534221
F. laevipes Spach (2)		Spain: Almería, Cabo de Gata	36º43’N/2º11’W	JGs/n-3 BdB 17	KJ534172	KJ534116	KJ534222
F. laevipes Spach (3)		Spain: Valencia, Tavernes de Valldigna, Fontetes de Cantus	39º4’N/0º16’W	ECA162, VAL203637	KJ534173	KJ534117	KJ534223
F. laevis Sennen (1)	ABCD	Tunisia: Nabeul Governorate, Korbous	36º49’N/10º34’W	AQ3297, VAL201248	KJ534168	KJ534111	KJ534217

F. laevis Sennen (2)		Spain: Valencia, Chella	39º1’N/0º41’W	JG4042, VAL181311	-	KJ534112	KJ534218
F. laevis Sennen (3)		Greek: Corinth Mesi Synoikia Trikalon	38º0’N/22º28’W	AH3517, VAL191287	KJ534169	KJ534113	KJ534219
F. laevis Sennen (4)		Spain: Cuenca, Alarcón	39°32’N/2°5’W	JGs/n-4 BdB 567	KJ534170	KJ534114	KJ534220
F. paphlagonica Bornm. & Janch. (1)	D	Turkey: Karabük Safranbolu, canyon Incekaya	41º16’N/32º41’W	CA6322, MA688598	KJ534174	KJ534118	KJ534224
F. paphlagonica Bornm. & Janch. (2)		Turkey: Çankırı	40º31’N/33º36’W	123PV06, MA774870	KJ534175	KJ534119	KJ534225
F. paradoxa Heywood in Guinea (1)	A	Spain: Cazorla, Puertollano	37°46'N/2°58'O	JG s/n, VAL8951	KJ534176	KJ534120	KJ534226
F. paradoxa Heywood in Guinea (2)		Spain: Jaen, Huelma, Sierra Magina	37º43’N/3º28’W	JG s/n, VAL206642	-	KJ534121	KJ534227
F. paradoxa Heywood in Guinea (3)		Spain: Cuenca, Tragacete, Puerto de Cubillo	40º19’N/1º46’W	JG4219, VAL207025	KJ534177	KJ534122	KJ534228
F. paradoxa Heywood in Guinea (4)		Spain: Jaén, Cazorla, Los Arenales	37º56’N/2º52’W	JG s/n, VAL26606	KJ534178	KJ534123	KJ534229
F. procumbens Gren. & Godr. (1)	ABCDE	Spain: Castellón, Fredes, Portell de l´Infern	40º42’N/0º11’W	JG4095, VAL181312	KJ534179	KJ534124	KJ534230

F. procumbens Gren. & Godr. (2)		Greek: Ioannina Flampourar	39º51’N/20º59’W	FJC716, VAL190471	KJ534181	KJ534128	KJ534234
F. procumbens Gren. & Godr. (3)		Armenia: Syunik Tatev, Devil´s Bridge	39°23’N/46°15’W	RG78, VAL177315	KJ534180	KJ534125	KJ534231
F. procumbens Gren. & Godr. (4)		Portugal: Bragança, Mogadouro, Bemposta	41º17’N/6º28’W	CA17820, VAL203793	-	KJ534126	KJ534232
F. procumbens Gren. & Godr. (5)		Spain: Jaén, GR, Hoyo Frío	37º43’N/3º28’W	JGs/n-5 BdB 570	-	KJ534127	KJ534233
F. procumbens Gren. & Godr. (6)		Turkey: Sivas Gürün	38º43’ N/37º17’W	FM4586, MA688958	KJ534182	KJ534130	KJ534236
F. procumbens Gren. & Godr. (7)		Turkey: Karabük , Safranbolu	41º16’N/32º41’W	CA6277, MA688761	-	KJ534131	-
F. procumbens Gren. & Godr. (8)		Morocco: Meknès-Tafilalet, Aït Aomar	32º36’N/4º48’W	SC18067, MA745059	KJ534183	KJ534132	KJ534237
F. procumbens Gren. & Godr. (9)		Greek: Kozani Palaiokastro, Mts. Vourinos	40º11'N/21º38'W	FJC856, VAL190473	-	KJ534129	KJ534235
F. procumbens Gren. & Godr. (10)		Bulgaria: Nova Lovcha	41º28’N/23º45’W	AQ1253, VAL163135	KJ534150	KJ534092	KJ534198
F. procumbens Gren. & Godr. (11)		Greek: Grevena, Varis	40º8’N/21º37’W	FJC778, VAL201699	KJ534151	KJ534093	KJ534199
F. procumbens Gren. & Godr. (12)		Greek: Achaea, Aghia Varvara, Mt. Ghaidhourorachi,Neraidorachi	37°58’N/22°18’W	JC0811, VAL190953	KJ534154	KJ534096	KJ534202

F. scoparia Pomel (1)	ABCD	Tunisia: Kasserine Governorate, Dashrat Zawiyat Sidi Salis, Djebel Chambi	35º12'N/8º40'W	JC3255, VAL201884	KJ534184	KJ534133	KJ534238
F. scoparia Pomel (2)		Spain: Valencia, Serra, Porta-Coeli	39º38'N/0º28'W	ECA160, VAL203636	KJ534185	KJ534134	KJ534239
F. scoparia Pomel (3)		Morocco: Meknès-TafilaletAït, Aomar	32º36'N/4º48'W	SC18033, MA745867	KJ534186	KJ534135	KJ534240
F. thymifolia Spach (1)	ABCD	Spain: Valencia, Bolbaite, Canal de Navarrés	39º1'N/0º41'W	JG4043, VAL181313	KJ534190	KJ534139	KJ534244
F. thymifolia Spach (2)		Tunisia: Nabeul Governorate, Korbous	36º50'N/10º34'W	AQ3311, VAL201344	KJ534189	KJ534138	KJ534243
F. thymifolia Spach (3)		Spain: Cádiz, Grazalema, Puerto de las Palomas	36º47'N/5º22'W	JG4193, VAL189047	KJ534187	KJ534136	KJ534241
F. thymifolia Spach (4)		Cyprus: Paphos, Dhrousha, Akamas peninsula, Turtle Bay	34º57'N/32º18'W	JRV5604, VAL189001	KJ534188	KJ534137	KJ534242
F. thymifolia Spach (5)		Spain: Cádiz, Barbate, Torre del Tajo	36º10'N/5º58'W	JG4187, VAL189058	KJ534166	KJ534109	KJ534215
F. trisperma Hub. - Mor. & Reese (1)	D	Turkey: Sivas Gürün	38º43'N/37º17'W	FM4585, VAL146758	KJ534191	KJ534140	KJ534245
Cistus L.							
C. albidus L.		Spain: Valencia, Serra, Porta-Coeli	39º40'N/0º28'W	MEs/n-1	KJ534141	KJ534083	-

C. clusii Dunal		Spain: Valencia, Serra, Porta-Coeli	39º39’N/0º29’W	MEs/n-2	KJ534142	KJ534084	-
C. creticus L.		Greek: Ahaia, Akrata, Zarochla	37º58'N/22º17'E	CN7078,VAL190419	KJ534143	KJ534085	-
Hopea Roxb.							
H. nervosa King					AY026651.1 GI:22034171	AB006384.1 GI:4210570	EF660015.1 GI:157272096
H. wightiana Wall.					AY026656.1 GI:22034176	AB246461.1 GI:94966593	EF660026.1 GI:157272107
Neobalanocarpus P.S.Ashton							
N. heimii (King) P.S.Ashton					AY026657.1 GI:22034177	AB006383.1 GI:4210568	EF660032.1 GI:157272113

Table 3: Characteristics of DNA regions used in the phylogenetic analyses of the *Fumana* accessions.

	ITS	*matK*	*TrnT-L*
Length rang (bp)	680-791	926-951	644-770
Aligned length (bp)	817	971	850
Number of variables/informative character	136 / 83	81/ 54	118/68
Maximum sequence divergence (K-2-p)	0.031 (3.1%)	0.033 (3.3%)	0.051 (5.1%)
CI	0.704	0.923	0.917
RI	0.884	0.981	0.977
Mean G+C content	57.6%	33.1%	30.7%
Substitution Model	TIM3+G	GTR+G	TVM+G
Simplest Model	GTR+G	GTR+G	GTR+G

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Supplementary Table 1: Comparsion of historical taxonomic treatments of *Fumana* using taxa names as published in original publications.

Dunal (1824)	**Spach (1836)**	**Willkomm (1857-1863)**
Helianthemum		
Sec. Fumana	Fumana	Fumana
		Sec. Eufumana Willk.
H. arabicum	F. arabica	Fumana arabica var. genuina
		Fumana arabica var. parviflorum
H. ericoides	F. vulgaris var. major	Fumana ericoides
H. fumana	F. vulgaris var. minor	Fumana spachii
H. procumbens		Fumana procumbens
		Sec. Helianthemoides Willk.
H. laevipes	F. laevipes	Fumana laevipes
H. laeve	F. viscida var. longifolia	Fumana viscida var. laevis
H. viride		Fumana viscida var. viride
H. juniperinum		Fumana viscida var. juniperina
H. barrelieri		Fumana viscida var. barrelieri
H. thymifolium	F. viscida var. thymifolia	Fumana viscida var. genuina
H. glutinosum		
		Fumana viscida var. papilosa

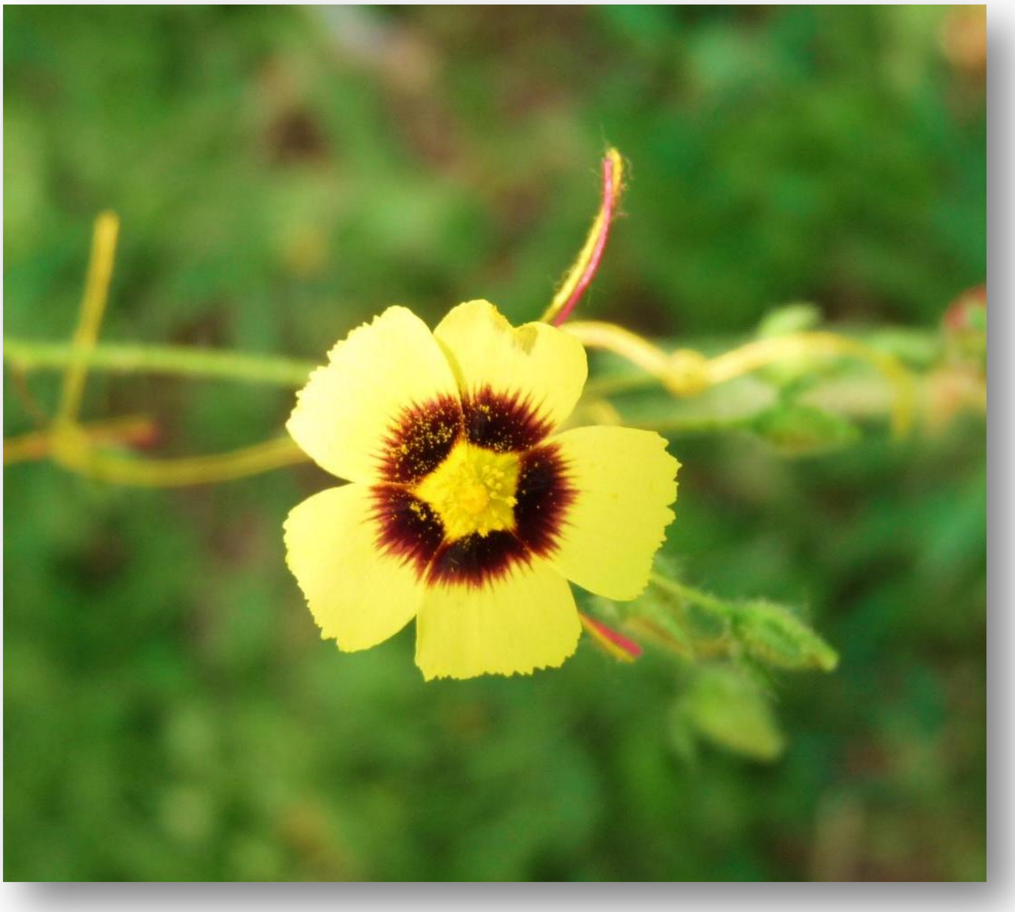
Continuation of supplementary table 1

Grosser (1903)	**Janchen (1920)**	**Coode (1965, only**	**Güemes & Molero**

<table>
<tr><td></td><td></td><td>turkish species)</td><td>(1993)</td></tr>
<tr><td></td><td></td><td></td><td></td></tr>
<tr><td>Fumana</td><td>Fumana</td><td>Fumana</td><td>Fumana</td></tr>
<tr><td></td><td colspan="2">Subgen. I Eufumana (Willk.) Janch.</td><td>Subgen. I Fumana</td></tr>
<tr><td></td><td colspan="2">Sec. 1. Platyphyllon Janch.</td><td></td></tr>
<tr><td>Fumana arabica var. genuina</td><td>Fumana arabica f. viridifolia</td><td colspan="2">Fumana arabica var. arabica</td></tr>
<tr><td>Fumana arabica var. incanescens</td><td>Fumana arabica f. incanescens</td><td colspan="2">Fumana arabica var. incanescens</td></tr>
<tr><td></td><td colspan="2">Sec. 2. Leiosperma Janch.</td><td></td></tr>
<tr><td></td><td></td><td></td><td></td></tr>
<tr><td>Fumana ericoides var. typica</td><td colspan="2">Fumana ericoides f. typica</td><td>Fumana ericoides</td></tr>
<tr><td>Fumana ericoides var. grandiflora</td><td colspan="2">Fumana ericoides f. grandiflora</td><td></td></tr>
<tr><td>Fumana ericoides var. montana</td><td colspan="2">Fumana ericoides f. montana</td><td>Fumana ericifolia</td></tr>
<tr><td></td><td>Fumana ericoides f. glandulosa</td><td>Fumana scoparia</td><td>Fumana scoparia</td></tr>
<tr><td>Fumana procumbens</td><td>Fumana vulgaris f. typica</td><td>Fumana procumbens</td><td>Fumana procumbens</td></tr>
<tr><td></td><td colspan="2">Fumana vulgaris f. alpina</td><td></td></tr>
<tr><td></td><td>Fumana vulgaris f. paphlagonica</td><td colspan="2">Fumana paphlagonica</td></tr>
<tr><td></td><td></td><td></td><td>Fumana baetica</td></tr>
<tr><td></td><td></td><td></td><td>Fumana paradoxa</td></tr>
<tr><td></td><td></td><td></td><td>Fumana lacidulemiensis</td></tr>
<tr><td></td><td></td><td></td><td>Subgen. II Pomelina Maire</td></tr>
<tr><td>Fumana calycina</td><td>Fumana calycina</td><td></td><td>Fumana fontanesii</td></tr>
<tr><td></td><td colspan="2">Subgen. II Fumanopsis (Pomel) Janch.</td><td>Subgen. III Fumanopsis (Pomel) Janch.</td></tr>
<tr><td></td><td colspan="2">Sec. 3. Helianthemoides Willk.</td><td></td></tr>
<tr><td>Fumana laevipes</td><td>Fumana laevipes</td><td></td><td>Fumana laevipes</td></tr>
<tr><td></td><td colspan="2">Fumana thymifolia f. laevis</td><td>Fumana laevis</td></tr>
<tr><td colspan="2">Fumana thymifolia f. viridis</td><td colspan="2">Fumana viscida var. viridis</td></tr>
<tr><td colspan="2">Fumana thymifolia f. juniperina</td><td></td><td>Fumana juniperina</td></tr>
<tr><td colspan="2">Fumana thymifolia f. barrelieri</td><td></td><td></td></tr>
</table>

Fumana thymifolia f. typica	Fumana thymifolia f. glutinosa	Fumana thymifolia var. thymifolia	Fumana thymifolia
Fumana thymifolia var. papillosa	Fumana thymifolia f. papillosa		
			Fumana hispidula
	Sec. 4. Megalosperma Janch.		
Fumana grandiflora	Fumana grandiflora	Fumana grandiflora	
	Fumana bonapartei		
Fumana oligosperma	Fumana oligosperma	Fumana oligosperma	
Fumana aciphylla	Fumana aciphylla	Fumana aciphylla	
		Fumana trisperma	

Trends of seed mucilage evolution in Mediterranean Cistaceae



CHAPTER 5

Abstract

Mucilage secretion by diaspores occurs in at least 83 Angiosperm families and it has been recently proposed that it is a derived character in the phylogeny of Angiosperms. The family Cistaceae provides us an opportunity to study the evolution of this character because the large variability of mucilage occurrence and their characteristics in genera and species within this family and the availability of a well solved phylogeny.

We use a phylogenetic approach to study mucilage seed evolution and to test the hypothesis that the presence of seed mucilage in the Cistaceae is an ancestral character state related to the ability of species to colonize eroded slopes. To achieve these objectives we mapped the presence of the character seed mucilage along the phylogeny of the Cistaceae, and analyse the relation of this character with traits associated to the competitive ability of the plants, such as relative growth rate (RGR), seed longevity and seed size of seven representative species of the family and discuss the results in the light of the environmental changes that occurred along the history of the family.

Seed mucilage secretion in the Cistaceae is the most likely ancestral character state and when the type of mucilage (cellulosic and pectin) was included in the analysis, we found that pectin mucilage seems to be the ancestral character state in the family. Seed mucilage was lost several times throughout the family but the most noticeable change is the total loss in the genus of *Cistus*. A pattern was found in the Cistaceae from low competitive species with strong mucilage secretion of cellulosic type, low RGR values, short seed longevities and big seed sizes that characterize the oldest genus (i.e. *Fumana*) towards the most modern species with a higher competitive ability, no mucilage secretion, higher RGR and seeds with longer viability and smaller sizes in the species of *Cistus*.

1. Introduction

Mucilage secretion by the testa of seeds (myxospermy) or the pericarp of fruits (myxocarpy) is a common attribute in many families of Angiosperms (Grubert, 1974; Grubert, 1981). Mucilaginous diaspores secrete a mucilage envelope once they come in contact with water (Werker, 1997) as a result of light rain, dew or even soil moisture (Huang et al., 2008). This mucilage absorbs a considerable amount of water in a short time and has been studied in relation to seed dispersal and germination processes. The accumulation of water can accelerate seed germination in some species (Gat-Tilman, 1995; Gutterman and Shem-Tov, 1996; Huang, Gutterman and Hu, 2000; Kreitschitz, Tadele and Gola, 2009) or delay it in some others as it makes seeds impermeable to gases (Atwater, 1980; Gutterman and Shem-Tov, 1996; Gutterman, Witztum and Evenari, 1967; Witztum, Gutterman and Evenari, 1969). Also, mucilage has been related to the repair of embryo DNA in desert conditions through its ability to hold water and to transfer it to the embryo in the first phase of seed rehydration (Huang et al., 2008; Yang et al., 2011). In addition, diaspores that secrete mucilage are strongly glued to the soil surface once the mucilage has dried completely. This phenomenon has been proven to minimize seed collection by ants (Engelbrecht and García-Fayos, 2012; Gutterman and Shem-Tov, 1997; Yang et al., 2013) and to reduce seed losses by soil erosion (García-Fayos and Cerdà, 1997; García-Fayos, Engelbrecht and Bochet, 2013; Han et al., 2011). Mucilage secretion in diaspores probably cannot be reduced to one single function for all species. It is likely that mucilage secretion in certain plant groups fulfils various functions at the same time yet integrated studies examining several functions are still lacking.

Two categories of mucilage have been described in relation to their composition and properties, “true” mucilage, which is in most species almost exclusively formed of pectin, and “cellulosic” mucilage, with the additional presence of a skeleton of cellulosic fibrils (Kreitschitz, 2009; Kreitschitz and Vallès, 2007; Mühlethaler, 1950; Werker, 1997). However, in some species the “true” mucilage can also contain hemicellulose. Differences in composition of the mucilage have been hypothesised to change its functionality, since cellulosic threads added structural strength to the pectin mucilage thus increasing the degree of adhesion and anchorage of the diaspore (simple staining reactions help to visualize and differentiate between both types; Grubert, 1974; Engelbrecht, Bochet and García-Fayos, 2014; Kreitschitz, 2009; Kreitschitz et al., 2009; Kreitschitz and Vallès, 2007; Western et al., 2000).

Mucilage secretion by diaspores has been reported to occur in at least 83 Angiosperm families Grubert (1974; Grubert, 1981, personal data). Yang (2012), after reviewing the available evidences proposed that mucilage secretion could be a derived character in the phylogeny of Angiosperms. However, in their review they did not take into account that mucilage secretion in diaspores is not a constant character throughout plant orders, families and even genera within families and, therefore, comprehensive data and analysis are still lacking.

In the present study we focus on the family of the Cistaceae, as a large variability of mucilage occurrence and quality in seeds can be found and the family phylogeny has been recently solved (Guzmán and Vargas, 2009). Examining the patterns of variation in the character mucilage secretion across the family Cistaceae in the context of the phylogeny, paleoenvironmental changes and the relationships between mucilage secretion and plant traits related to its colonization ability could help us to determine the history and function of seed mucilage secretion in this plant linage.

The family of the Cistaceae consists of 8 genera with about 180 species and is one of the most characteristic plant families living in open and degraded shrubland of European-African Mediterranean ecosystems. (Guzmán, Lledó and Vargas, 2009; Thompson, 2005). Seed mucilage has been found in 7 out of 8 genera in the family and it is only completely missing in the genus *Cistus* (Appendix; Muñoz Garmendia and Navarro, 1993). Furthermore, the occurrence of species with seed mucilage varies between genera, from *Fumana* and *Helianthemum,* which have a very high proportion of species with mucilaginous seeds to *Halimium* and *Lechea*, which have a very low proportion of species. There is almost no information about the type of mucilage in this family. Interesting, the only study on this topic found differences in mucilage composition between phylogenetically near genera (Engelbrecht et al., 2014) with cellulosic mucilage in *Fumana ericifolia* but pectin mucilage in *Helianthemum violaceum* (Engelbrecht et al., 2014).

A recent phylogeny of the family of the Cistaceae has been established (Guzmán and Vargas, 2005; Guzmán and Vargas, 2009) revealing that *Fumana* is a basal genera in the family that appeared during middle Miocene (18.51–10.17 Ma) while *Cistus* is the most recent genus, with the formation of the *Tuberaria-Halimium-Cistus* clade appearing during the Pliocene (5.3-4.22 Ma; Guzmán and Vargas, 2005) and a posterior radiation of *Cistus* during the Pleistocene (Guzmán et al., 2009). The main diversification centre of the Cistaceae is proposed to be the Mediterranean Basin (Guzmán and Vargas, 2009).

Putting together all these data emerged the suggestion that mucilage is an ancestral character in this family, which has been lost in the most recently evolved genera.

The function of seed mucilage secretion has been recently studied in *Helianthemum* and *Fumana* species, emphasizing its importance as an antitelechoric dispersal mechanism that reduces seed loss through runoff as well as it diminishes seed predation by granivorous ants (Engelbrecht and García-Fayos, 2012; García-Fayos, Bochet and Cerdà, 2010; García-Fayos et al., 2013). Soil erosion and water runoff along slopes are frequent phenomena in arid and semi-arid regions of the Mediterranean, in which most of the annual rainfall is concentrated into a small number of high intensity events (Thompson, 2005). As a consequence, high soil erosion occurs on steep slopes (Poesen and Hooke, 1997), and diaspores on the soil surface are at a risk of being removed downslope with runoff water and deposited in the lower parts of the slopes or in the valley bottoms where they can get buried (García-Fayos et al., 1995) or where seedling establishment and development can be affected by stronger competition than on the slopes. In consequence, anchoring the diaspore to the ground by adherence mechanisms can be a favourable adaptation to remain on the slopes and to avoid strong competition with other plants.

Small seeds are more prone to be removed by soil erosion than bigger seeds (Cerda and Garcia-Fayos, 2002). In addition, in most of the temperate floras small seeds have been associated to high seed persistence (Leishman et al., 2000) but also that competition between seedlings, and particularly between siblings, favours seed persistence (Siewert and Tielborger, 2010; Thompson, 2000). On the other hand, a faster and larger plant growth positively influences survival in competitive environments, as other plants can be outgrown, and plant growth immediately after seed germination is dependent on the specific relative growth rate (RGR; Turnbull et al., 2008). Therefore, we predict that a gradient of mucilage occurrence must be found in relation to other gradients of decreasing seed size and increasing seed persistence and RGR in the species of Cistaceae.

We use a phylogenetic approach to study mucilage seed evolution and to test the hypothesis that the presence of seed mucilage in the family of the Cistaceae is an ancestral character state related to the ability of species to colonize eroded slopes. We also explore the association between the ability of seeds to secrete mucilage and plant traits related to the competitive ability of species. To achieve these objectives we mapped the presence of the character seed mucilage along the entire phylogeny of the Cistaceae, and analyse the relation of this character with the relative growth rate (RGR), seed longevity and seed size of some representative species of the family, then discussing all it in the light of environmental changes that occurred along the history of the family.

2. Material and Methods

2.1 Ancestral character state reconstruction

To detect if seed mucilage secretion in the Cistaceae is an ancestral character state, we first tested for its presence in seeds of all genera and as much species per genera as possible. We used seeds from field samples and from seed banks and also collected information from the literature. Tests were carried out by fully submerging the seeds in distilled water during at least 3 hours and then checked under microscope for the presence of a mucilaginous envelope. To differentiate between pectin and cellulosic mucilage, seeds whose mucilage has been secreted were stained with methylene blue for 1 to 3 hours to detect cellulosic strands.

We used a reduced version of the phylogenetic tree of the family of the Cistaceae published by Guzmán et al. (2009) maintaining the same branch lengths but pruning the tree with the “drop.tip” command of the package “ape” (Paradis et al., 2013) in R version 3.0.1 (R Development Core Team, 2011) to keep only species for which mucilage character state could be determined.

To estimate the evolutionary pathways of mucilage secretion in seeds in the family of the Cistaceae, we used three different approaches. As a first approach we ran a maximum likelihood character reconstruction and as second approach a parsimony character reconstruction in the program Mesquite v.2.74 (Maddison and Maddison, 2009) to study presence and absence of mucilage secretion in the seeds. Species of Cistaceae where traced onto the pruned phylogenetic tree of Guzmán (2009), using the “Trace Character History” option under the parsimony and likelihood reconstruction method of the program Mesquite v.2.74 (Maddison and Maddison, 2009). The maximum likelihood model includes information from genetic branch lengths and used the Markov *k*-state 1 parameter model (Mk1), which assumes a single rate for all transitions between states.

As a third approach, we ran a Bayesian binary Markov chain Monte Carlo (MCMC) approach implemented in RASP (Reconstruct Ancestral State in Phylogenies) version

2.0 beta (Yu, Harris and He, 2010), an updated version of S-DIVA (Yan et al.,2010), to analyse the different types of mucilage (pectin mucilage, cellulosic mucilage or absence of mucilage). We ran 5000 000 MCMC cycles with 10 chains, a temperature of 0.1 and a fixed JC + G (Jukes-Cantor + Gamma) with null root distribution and equal rates. The maximum number of areas for this analysis was kept as 3. RASP was designed for phylogeographical analyses, but the methodology is appropriate for other traits if these are discrete or polymorphic. RASP determines the probabilities of each character state for each node. In our analysis we used the consensus BI tree (Guzmán and Vargas, 2009) and we did not allow the internal nodes to be polymorphic.

2.2 Mucilage and competitive ability relationships

We used species of 5 different genera of the family (*Cistus populifolius*, *C. monspelliensis*, *Fumana ericoides*, *F. leavipes*, *Helianthemum syriacum*, *H. violaceum*, *Halimium halmifolium* and *Tuberaria lignosa*) for the experiments described below. Species ranged from herbaceous plants to small shrubs and are representative of seed mucilage response for each genus in the family. Seeds were collected in the summer of 2009 at Sierra Calderona, 25 km north of Valencia, Spain (39°39'26N 0°28'49E) except for seeds of *Halimium halmifolium*, which were collected at El Saler, 10km south of Valencia (39°21'34N 0°19'31E), and those of *Cistus monspeliensis* collected at Teruel (40°20'13N 1° 9'30E) in the same dates.

2.2.1 Relative growth rate (RGR)

Seed weight of all experimental species was obtained by weighting 50 fully mature seeds individually to the nearest 0.01 mg with a precision balance (Mettler Toledo AX 205), except seeds of *Tuberaria lignosa*, which were weighted in 50 lots with 10 seeds each. Seeds were pretreated and germinated following Moreira (2010). Seedlings were transferred to prepared pots (10x10cm), which were filled with a mixture of coconut and peat fibers. Plants were grown in a greenhouse for 56 days (8 weeks), from January till March of 2012, with temperatures ranging between 6° C and 30°C. Pots were watered daily and their location in the greenhouse was rearranged every week to homogenize temperature and light conditions for all plants. On day 56, plants were removed from the pots and the roots cleaned with water. Remaining soil particles were carefully removed from roots by hand in the laboratory. Leaves and roots were then air dried at 80°C for 12 hours and weighted directly afterwards with a precision balance (Mettler Toledo AX 205). The final RGR per species was calculated as the mean of the ratio of plant to seed weights expressed in grams and was used as a surrogate for the speed that seedlings can growth and then potentially overtake and overtop its competitors.

2.2.2 Seed longevity

Twenty four sets of 25 fully mature seeds of the selected species were mixed with sand (pH: 9.4; Carbonates (%) :<0.5) and placed in 5×5cm nylon bags of fine mesh that avoided seed losses but allowed the circulation of water and air. The bags were then buried in the same sand at a depth of 10cm in a block design at the CIEF (Autonomous Government of Valencia) experimental site in Quart de Poblet. Each block consisted in one bag per species. Seeds were buried on November 2009 and then 6 bags with 25 seeds per bag (150 seeds per species) were recovered in November of years 2010, 2011 and 2012.

After extracting the bags from the soil, we brought them to the laboratory and then sieved the content of each individual bag until the immediate greater particle size than seeds of the species contained at that bag and then recovered the seeds from the remaining sand using a microscope. Intact and germinated seeds were recognized and the intact seeds checked for viability. Intact seeds were soaked in water for 12 hours, then the testa cut under a binocular, and afterwards immersed in a 1% Tetrazolium chloride (TTC) solution in the dark for 24 hours at 25ºC. Embryo and cotyledon were checked for strong pink staining using a binocular. Very light staining as well as small stained spots on the embryo were not counted as viable seeds (Peters, 2000). The ratio of seed survival per bag (viable seeds recovered per bag/viable seeds per bag) was used to estimate mean seed viability for each species and year. Then, we fitted linear models of seed viability decay through time per species and the slope of the linear model was used as a surrogate of seed persistence.

2.2.3 Statistical Analysis

ANOVA analysis was used to analyse the relationship between the type of mucilage (pectin or cellulosic) as predictor variable and relative growth rate (RGR), seed persistence (slope of seed viability decay) and seed size (mean seed weight) of the species as response variables. To calculate pairwise comparisons between group levels, post hoc analyses with the p-value adjusted by Bonferroni method were performed. Statistical analyses were carried out with the “lm” and the “pairwise.t.test” commands in R version 3.0.1 (R Core Development Team, 2011).

3. Results

3.1 Ancestral Character State

We found that all the 21 tested species in the genus *Fumana* have seeds that secrete mucilage on wetting (Engelbrecht et al., Submitted). Likewise, all the seeds in the genus *Helianthemum* and *Tuberaria* used in the phylogenetic tree of Cistaceae by Guzman (2009) secreted mucilage on wetting and only 5 of 60 additionally tested species in the genus *Helianthemum* (110 sp.) were found to not secrete mucilage. Eight species in the genus *Halimium* (9 sp.) showed no mucilage secretion when tested with exception of one species (*Halimium umbellatum*) for which a positive result was found in the literature (Grubert, 1974). However, *Lechea tripetala* as well as 7 of 13 additionally tested species of the genus *Lechea* (17 sp.) do not secrete mucilage while mucilage was completely absent in all species tested of the genus *Cistus* (18 tested of 20 sp.). Number of species per genera in *Cistus* follows Guzmán and Vargas (2005) Engelbrecht (Submitted) for *Fumana* and for the rest of the genera information was extracted from Mabberley (1997).

Mucilage secretion on wetting in seeds of the Cistaceae resulted in the most likely ancestral character state in the parsimony, likelihood and Bayesian analysis (1, 0.80 and 0.99 respectively). We only display the results of the Bayesian analysis as results are similar (Figure 1).

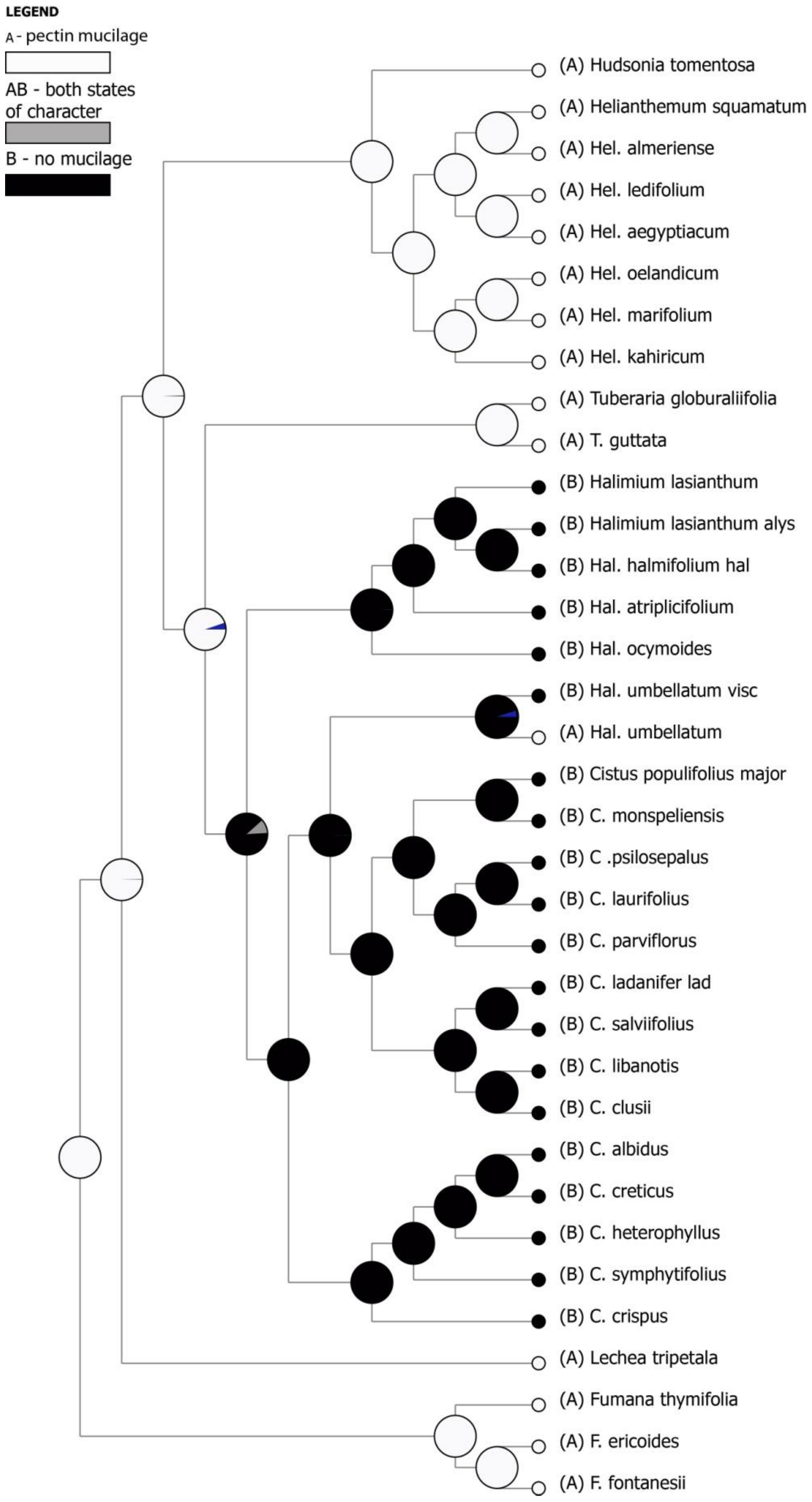


Figure 1: Graphical results of ancestral state reconstructions for mucilage secretion obtained by RASP (S-DIVA). Presence of pectin mucilage (A) and absence of mucilage (B) is analyzed at each node of the phylogeny of the family of the Cistaceae (Guzman, 2009) Pie charts represent Bayesian credibility values

and the legend represents colour key to possible ancestral character states at different nodes; with blue represents other ancestral ranges.

We found that *Fumana* seeds have cellulosic type mucilage, with cellulosic fibrils embedded in the pectin coat, while *Helianthemum*, *Tuberaria* and *Halimim halmifolium* seeds have mucilage of the pectin type only. We could not test the seeds of *Lechea* for mucilage type for the present paper, but after revising the detailed drawings after the mucilage tests performed in our laboratory in 1998 (Ana Vila and García-Fayos, unpublished data), we were able to assign it to the pectin mucilage type.

When mapping the type of mucilage (cellulosic, pectin and absence of mucilage) along the phylogenetic tree, the Bayesian analysis in RASPs shows pectin mucilage (A) as the most likely ancestral character state (0.899), with a small probability of cellulosic mucilage (AC) as ancestral state (0.086) and a minor fraction of other combinations and the absence of mucilage (B, 0.0001). The parsimony analysis also reveals pectin mucilage as significant most parsimony ancestral character state (1.00) while the likelihood analysis shows as significant most likely ancestral state for the pectin mucilage (0.69) followed by the cellulosic mucilage type (0.27).

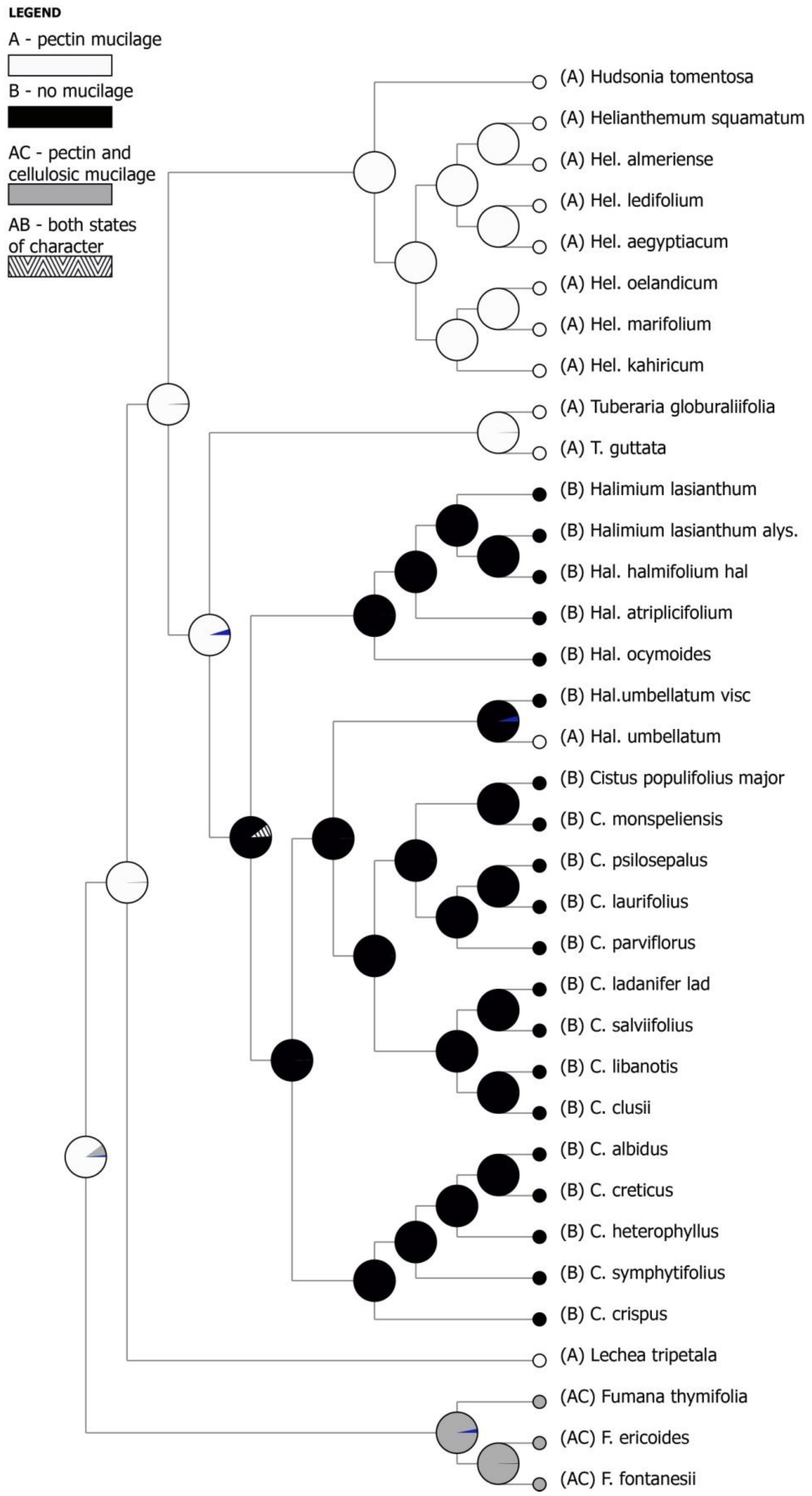


Figure 2: Graphical results of ancestral state reconstructions for mucilage secretion obtained by RASP (S-DIVA). Presence of pectin mucilage (A), absence of mucilage (B) and pectin with cellulosic mucilage (AC) is analyzed at each node of the phylogeny of the family of the Cistaceae (Guzman, 2009) Pie

charts represent Bayesian credibility values and the legend represents colour key to possible ancestral character states at different nodes; with blue represents other ancestral ranges.

3.2 Relative growth rate (RGR)

We found the largest relative growth rate (RGR) in *Halimium halimifolium*, followed by *T. lignosa* (see Table 1). Both species show a value over 200 $g.g^{-1}$ after 56 days. *Cistus monspeliensis*, *C. populifolius* and *H. syriacum* show similar values, while we found a strong decline in *H. violaceum* plants. The lowest values were measured in *F. ericifolia* and *F. laevipes* (Table 1).

When comparing the seed weight of each species, *F. ericoides* followed by *F. laevipes* had the heaviest seeds (Table 1). *Cistus populifolius* showed heavy seeds as well, followed by *H. violaceum* and then *C. monspeliensis*. *Halimium halimifolium*, *T. lignosa* and *H. syriacum* (Table 1).

Table 1**:** Mean measured value and typical error of relative growth rate (RGR), seed longevity, decay of seed viability as well as presence and type of mucilage for the 7 studied species.

Species	**Cistus monspeliensis**	**C. populifolius**	**Halimium halmifolium**	**Helianthemum syriacum**	**H. violaceum**	**Fumana ericoides**	**F. leavipes**	**Tuberaria lignosa**
Mucilage	No	No	No	Yes	Yes	Yes	Yes	Yes
Mucilage type	absent	absent	absent	pectic	pectic	cellulosic	cellulosic	pectic
RGR (g.g^{-1})	198.07 ± 15.31	172.29 ± 19.572	267.23 ± 38.63	161.38 ± 25.2	84.19 ± 12.36	17.95 ± 1.22	29.37 ± 1.67	220.49 ± 44.51
Viability after 3 years (%)	77.33 ± 1.97	46.69 ± 3.42	13.33 ± 2.45	8 ± 2.06	67.48 ± 2.31	8 ± 2.53	2 ± 0.89	12.66 ± 4.11
Longevity decay (slope of function)	5.261	13.829	13.575	28.548	9.017	27.58	29.435	25.759
Dry seed weight (mg)	0.921 ± 0.027	1.298 ± 0.033	0.673 ± 0.019	0.351 ± 0.006	0.959 ± 0.036	2.389 ± 0.047	1.431 ± 0.017	0.373 ± 0.07

3.3 Seed longevity

Seed viability after three years was the highest in *C. monspeliensis*, followed by *H. violaceum*. Both species have also the lowest slope when fitting a linear model over the seed survival (Table 1). Almost 50 percent of *Cistus populifolius* seeds still survived after three years buried but only 13.33 percent of *Halimum halimif*olium seeds did. However, linear models in both species showed similar declining slopes of seed survival (Table 1). In *T. lignosa*, after three years, only 12 percent of the seeds still survived and the slope of the fitted linear model was relatively steep. *Helianthemum syriacum* and *F. ericifolia* had similar low seed survival percentage after three years and similar declining slopes. However, the lowest survival percentages were found in *F. laevipes* and *T. guttata*, with only 2 and 3 percent respectively of all seeds surviving after three years. These species also showed the steepest slope of seed survival declining (Table 1).

3.4 Mucilage and competitive ability relationships

We found a significant relationship between the type of mucilage secretion and RGR. Seeds without mucilage had significantly higher RGR values than seeds with mucilage of cellulosic type (*p-value*: 0.011).

The analysis of the relation between mucilage type and the slope of seed viability decay indicate that seeds with cellulosic mucilage had significantly higher viability decay than seeds with pectin mucilage and also than seeds without mucilage (*p-value*: 0.047). Likewise, seeds without mucilage had marginally significant lower viability decay compared with the other seeds (*p-value*: 0.051).

When we analysed mucilage type in relation to seed weight across species, we found that seeds with cellulosic mucilage had marginally significantly heavier seeds (*p-value*: 0.057) than seeds with pectin type and seeds without mucilage. Likewise, seeds with pectin type of mucilage were lighter than the rest of the tested seeds (Table 1) but only marginally significant lighter than seeds with cellulosic mucilage (post-hoc bonferroni *p-value*: 0.052).

4. Discussion

Seed mucilage secretion in the Cistaceae is the most likely ancestral character state when mucilage presence and absence was analysed along the phylogenetic tree. When the type of mucilage (cellulosic and pectin) was included in the analysis, we found that pectin mucilage seems to be the ancestral character state in the family. The evolution of the character in the family then started with a presumed ancestor whose seeds secrete pectin mucilage only, then the basal *Fumana* clade acquire the cellulosic mucilage character while only pectin mucilage was maintained in the remainder clades and in several of them tended to a progressive loss of mucilage secretion (Figures 1 and 2). The loss of seed mucilage seems to have occurred three times in the history of the Cistaceae, in genus *Lechea*, *Hudsonia* and *Cistus* (Figure 1). However, the phylogenetic tree does not include all species of the family and some additional variation may be lost. In this sense, we also report three additional losses of mucilage character within the genus *Helianthemum*, *Halimium* and in species of *Lechea* differing from the one included in the phylogenetic analysis (see section 2.1). The loss of seed mucilage in species of *Helianthemum* and *Halimium* occurred at the same time frame that the diversification of genus *Cistus* (Pliocene- Pleistocene, Guzmán and Vargas, 2009) and it can be assumed that similar climatic and vegetation conditions could have triggered these changes in the seeds. In *Lechea*, dating information is lacking and hence, no conclusions can be drawn. The pattern we found that mucilage secretion is an ancestral character in the Cistaceae family put into question the proposal by Yang et al. (2012) who stated that mucilage is an evolutionary advanced state character because they found it more frequently in phylogenetically advanced families of Angiosperms.

The dated phylogenetic tree estimates the appearance of Cistaceae to have occurred 29-22 Ma ago (Guzmán and Vargas, 2009) during the Oligocene, with *Fumana* being the most ancestral genus as it branched up first in Middle Miocene (18.51-10.7 Ma). It was an age of global expansion of Angiosperms after the notable extinction event that occurred at the start of Oligocene. Middle Miocene represents one of the last warm episodes of the Neogene (Miocene Climatic Optimum, MCO). In a general cooling trend, it was an age when desert conditions expanded against tropical forests and new niches were available for plants (Axelrod, 1975; Jiménez-Moreno, Fauquette and Suc, 2010; Jiménez-Moreno and Suc, 2007). Oligocene was also a time of strong tectonic changes and important erosion processes occurred in western Mediterranean Basin when Alps started to rise in Europe (Alpine Orogeny; Barrón et al., 2010). All these conditions might favour plant species that were able to colonize and survive in open areas with low plant competition and stressful conditions, like *Fumana*.

Cistaceae branched up further in the Middle Miocene towards the end of the Miocene (Barrón et al., 2010) with the appearance of the *Hudsonia-Helianthemum* clade (9.20-5.15 Ma) and the loss of the cellulosic type of mucilage, but maintaining the pectin mucilage, at the same time that species largely increased their competitive ability (*H. syriacum*) and reduced seed size and seed survival (*H. violaceum*, see Table 1). Towards the end of Miocene and after the important cooling event that occurred 14.8-14.5 Ma ago, a predominance of herbs was already established in large parts of the Western Mediterranean Basin, and from the Pliocene a strong expansion of the grasslands and steppes occurred (Jiménez-Moreno et al., 2010) with a subsequent increase of wildland fires (Retallack, 2001). In this time period the *Tuberaria-Halimium-Cistus* clade appeared (Guzmán and Vargas, 2009). In consonance with this scenario, species of this clade also have high competitive ability and small seed sizes, like *Helianthemum-Hudsonia* clade, but at the same time species are characterized by an increase in seed longevity and by seeds whose germination is favoured by forest fires (Doussi and Thanos, 1994; Thanos et al., 1992). The subsequent radiation of genus *Cistus*, *Halimium* and *Helianthemum* from the end of the Pliocene and along the Pleistocene occurred after the onset of the Mediterranean climatic conditions 2.5 Ma ago, and has been related to the establishment of heterogeneous ecological conditions coexisting in the Mediterranean landscapes (Guzmán et al., 2009).

A pattern was found in the Cistaceae from species with strong mucilage secretion of cellulosic type, low RGR values, short seed longevities and big seed sizes that characterize the oldest genus (i.e. *Fumana*) towards the most modern species with no mucilage secretion, higher RGR and seeds with longer viability and smaller sizes in the genus *Cistus*. Species with mucilage secretion of pectin type (i.e. *Helianthemum*) showed intermediate values of these variables (see Table 1).

Models and empirical data show that large seeds have a better chance of success in competitive environments than smaller ones but also that large seeds are selected in environments under harsh environmental conditions, such as those with low but predictable precipitation or fertility (Turnbull et al., 2004; Volis and Bohrer, 2013). According to the tolerance-fecundity model, large seed sizes would be selected in plant colonization of stressful habitats (Muller-Landau, 2010), as has been found to explain the variability in seed size among populations of *Plantago coronopus*, even though variation in plant size and other life cycle components as additional strategy to cope with environmental variations were found as well (Villellas and García, 2012).

Bigger seeds have larger energy reserves to assist seedlings to produce more extensive root systems than smaller seeds, allowing them to obtain enough water and nutrients and conferring better establishment opportunities under stressful conditions in Mediterranean shrubland, as it has been reported for *Fumana ericoides* (Lloret, Casanovas and Peñuelas, 1999). In species with big seeds, mucilage secretion may be an additional advantage because it aids seeds to survive from seed collection by ants and the loss because soil erosion (Engelbrecht et al., 2014; Engelbrecht and García-Fayos, 2012). Likewise, models also showed that enhancing precipitation, increasing its unpredictability or introducing other perturbations (i.e. gap opening, drought events and forest fire) produce a selection towards decreasing seed mass and increasing dormancy as compared with more constant environments (Hodkinson et al., 1998; Leishman et al., 2000; Volis and Bohrer, 2013). The relative high RGR and the smaller size, higher longevity and physical dormancy of the non-mucilaginous seeds of *Cistus*, confer them an advantage to establish and survive in more competitive or frequently perturbed environments than do Cistaceae species with mucilaginous seeds. Additionally, because species producing small seeds can produce numerous seeds too, these species have large population growth rates that moreover increase intraspecific competition (Luis-Calabuig, Tárrega and Valbuena, 2000).

Despite these two extreme trends, models also show that there is a broad range of combinations of seed size and dormancy that result in similar fitness values under almost the full range of precipitation and environment predictability in Mediterranean conditions (Volis and Bohrer, 2013).

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Discussion

Our study on mucilage secretion by seeds showed clearly that myxodiaspory functions as antitelechoric mechanism in several species of the Cistaceae, as the mucilage effectively glued the seeds to the ground thus hindering further movement under simulated erosive conditions (drop impact and runoff) and preventing seed collection by granivorous ants. Regarding the evolutionary history of mucilage in the family of the Cistaceae, we found that the most ancestral character state is the presence of mucilage while the loss of mucilage is a derived character. The same pattern was found when focusing on the genus *Fumana*, in which a strong mucilage secretion was revealed to be the most ancestral state with a shift towards weak and absent mucilage in some of the more recent species.

Our results show that mucilage secretion works efficiently as an antitelechoric mechanism as proposed by Ellner and Shmida (1981) and Van Rheede van Oudtshoorn (1999) since the seeds of the tested species that were glued to the ground with their own mucilage survive longer to predation by granivorous ants and from being washed away by runoff and erosion than those seeds whose mucilage was prevented to be secreted.

In relation to the role of mucilage secretion in restricting seed predation by ants, our work goes further in the timeframe than previous experimental studies that captured only its effects during few days, which might not be enough time to guarantee that the mechanism effectively runs until seeds can germinate. In *F. ericoides*, seeds germinated between 3-24 month after being dispersed (Llorens et al., 2008) and in *F. thymifolia* typically between 4-5 month (50% germinated; Moreira et al., 2010) thus being exposed to seed collection in the season in which granivorous ants have their maximum of activity, from spring to late summer (Cerdá, Retana and Cros, 1998). In the present study, seeds of *F. ericoides* glued to the ground with their own mucilage maintained a 71% survival probability even after 3 month (91 days) and *F. thymifolia* 68% after 2.5 month (84 days), hence almost doubling the chance of survival for those seeds.

Ellner and Shmida (1981) proposed that antitelechory should not be considered an adaptation to aridity per se and the constraints imposed due to climate water shortage. Instead, it should be considered a side effect to the consequence of this aridity; that is, the chance of the seeds to be predated by animals or be removed by erosion processes in such open habitats. The frequency of plants with antitelechoric mechanism, such as myxospermy, would then be favoured in plant communities affected by soil erosion but it would not be affected by increasing aridity. Accordingly, we found higher frequency of antitelechoric species in plant communities of areas affected by erosive pressures in comparison to those communities of flat areas without soil erosion, but frequency of antitelechoric species was not influenced by the increase of aridity. In contrast, the proportion of species with atelechoric mechanisms (the absence of dispersal mechanism) increased slightly with dryness but was not influenced by soil erosion. These results agree with other results supporting the hypothesis that anchorage mechanisms in diaspores play a role in shaping species composition in communities affected by intense soil erosion.

However, mucilage secretion as antitelechoric mechanism could not only be a side effect but an adaptive mechanism to soil runoff per se (Cantón et al., 2004; García-Fayos et al., 1995). We tested the adaptive response of myxospermic seeds to soil erosion and expected to find two assumptions coming true: first, that intra and inter-population variation of this ability exists and that this variation should be related to the soil erosion pressure in which the plants live and second, that a higher amount of mucilage would be related to a higher soil adherence and then a higher resistance of the seeds to be removed by rain (drop impact and water runoff).

According to our hypothesis, we detected individual variation of seed mucilage among individuals within populations and among populations. The higher amount of mucilage was found in seeds collected from individuals that had developed under high soil erosion pressure. However, it only holds for *F. ericifolia* but not for *H. violaceum*, for which the differences were not significant. Also supporting our hypothesis, we found that a higher amount of seed mucilage was translated into a stronger and longer seed adherence under drop impact for one of the species studied (*F. ericifolia*) but not for the other species (*H. violaceum*). Consequently, the environment where the mother plant lives have a critical impact on the success of seedling establishment of *F. ericifolia*, as arid and semi-arid ecosystems are characterized by a sparse vegetation cover, which is often arranged as a two-phase mosaic of vegetated and bare ground patches that is under the control of water runoff and soil erosion (Valentin, 1994). Our results indicate that mucilage secretion can be an adaptive response, but seems to be species dependent. However, this species dependency response could be linked to the differences in the type of seed coat mucilage between the studied species. Till now little focus has been put on the structural differences in seed coat mucilage within a plant family. Here, we found pectin type mucilage in *H. violaceum* and cellulosic type mucilage in *F. ericifolia*, which furthermore behaved differently under run-off and drop-impact conditions. Seeds with cellulosic mucilage *of F. ericifolia* showed a stronger adherence (100% of seeds stayed glued during the experiment) in runoff experiments than the seeds with pectin mucilage of *H. violaceum* (60 % of seeds stayed glued), which could be a result of the assumed extra strength to the gluing abilities by the cellulosic strands (Grubert, 1974; Gutterman, Witztum and Evenari, 1967; Sullivan et al., 2011). Likewise, mucilage secretion of seeds of *F. ericifolia* related positively with their resistance to drop impact but seeds of *H. violaceum* did not have an obvious relationship.

When analysing the phylogenetic relations and mucilage occurrence in the family of the Cistaceae, we found that the presence of mucilage in seeds is the most likely ancestral character state with a tendency towards a loss of mucilage towards the more recently separated clades (*Cistus*, *Halimium*). As we assume that myxospermic species in this family are adapted to soil erosion processes, we can predict that life cycle strategies of those plants to colonize such environmental conditions should differ strongly to that of non-myxospermic species. Soil erosion is a geomorphologic process that increases both the stress and frequency of disturbances affecting plants and therefore tends to reduce plant cover and species diversity (Guerrero-Campo and Montserrat-Martí, 2000; Thornes, 1990). Life cycle strategies to cope with this environmental condition should then focus to provide seeds with enough resources to establish and survive in such poor conditions. On the contrary, species without seed mucilage secretion should be frequently removed to the downslope where they may compete with other conspecifics or other plant species. When focusing on live cycle strategies, we found a significant relation between the loss of seed mucilage and traits related to the competitive ability of the plant, as the relative growth rate (RGR), seed longevity and smaller seed size. This indicates an advantage to establish and survive in more competitive or frequently perturbed environments than species of the same plant family with mucilaginous seeds. This is coherent with the assumption of Grime (Grime, 1977; 1973), that nutrient rich and undisturbed habitats should have more species with high competitive abilities relative to species of disturbed and stressful habitats, such as erosive environments in the present case.

The phylogenetic history of the family of the Cistaceae emerges between the Miocene till the Pleistocene and climatic and ecological changes seem to have triggered diverse changes in plant traits (Guzmán, Lledó and Vargas, 2009; Guzmán and Vargas, 2009a; Guzmán and Vargas, 2009b), indicating that similar pressures could have provoked the changes in seed mucilage and competitive traits.

Analysing the ancestral character state of the genus *Fumana*, a similar tendency was found in which the most likely ancestral character state is a strong mucilage secretion with a trend towards a loss of mucilage in a more recently separated group of species. However, three of the four species the genus *Fumana* with less mucilage are found frequently in mountainous environment (Güemes, 1991) in which we expected a high amount of mucilage due to soil erosion pressure and the advantages of an antitelechoric mechanism. Interestingly, the change towards a loss of seed mucilage in these species is connected to a change in the dispersal abilities of the seed in general, changing from seed dispersed to fruit dispersed mode, retaining the seed inside of the capsule. As environments of these species are extremely rocky, we hypothesize that seed dispersal inside of the capsule bears higher chances to be rolled in a rocky fissure than a mucilaginous seed, which could be glued onto stones where seedling establishment would be impossible. In this case, the loss of seed mucilage is triggered by other factors than in the case of the family of the Cistaceae.

In conclusion, the family Cistaceae provides an interesting case to study the origin and function of seed mucilage from an ecological and evolutionary point of view. Our results offer evidence on the ancestral character of this trait and the environmental forces that shaped it. We also found support for our hypothesis that mucilage secretion in seeds in species of this family may be related to a successful colonization of open and frequently eroded habitats. We put on evidence that phenotypic variability exists in the amount of mucilage secreted by the seeds in some species and that this variability was related to the ability of these seeds to resist removal by erosive forces. However, trait heritability and how this variability translates to differential plant fitness in those environments remain untested. Further research is also needed in order to confirm this trend in other plant families living in semiarid conditions and also to deepen the knowledge of the connection between the different types of mucilage, their evolutionary history and their ecological functions.

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Conclusions

I. Mucilage secretion reduces seed removal produced by erosive processes which occur in natural conditions. These processes can be rain drop detachment and removal by water runoff.

II. The adaptive value of the mucilage secretion is species dependent as a relation between the amount of seed mucilage and adhesion power under rain drop impact was found for *Fumana ericifolia*, but not *for Helianthemum violaceum*.

III. The adaptive value of mucilage secretions seems to be mechanism dependent, as a relation in the amount of seed mucilage secreted proportional to the loss of seeds to rain drop impact was found, but not to the process of water runoff.

IV. The amount of seed mucilage in some species is influenced by the conditions in which the mother plant lives, as a positive relation between the strength of the erosion processes that plants experience and the amount of mucilage secreted by the seed was detected in *Fumana ericifolia*. However, the individual and not the population from which the seed was collected had the highest influence over the amount of mucilage produced.

V. The results indicate that an antitelechoric mechanism, such as myxodiaspory, are not adaptive to arid environments, as increasing aridity did not influence the frequency of its appearance. Furthermore, antitelechoric mechanisms seem to be favoured by surface runoff, as predicted by Ellner and Shmida (1981), since a higher frequency of species with those dispersal mechanisms under erosive conditions in comparison to non-erosive sites was found.

VI. Intense soil erosion can shape species composition at a community level as anchorage mechanisms in diaspores strongly influenced the species composition in communities affected by those mechanisms.

VII. The studied target species were extensively predated by diverse granivorous ants in the study area as flowers, calices, capsules and seeds were highly predated during the studied time period. Seed mucilage reduced the predation by granivorous ants significantly. When comparing seeds which were glued to the ground with their own previously secreted mucilage to lose seeds without mucilage, survival probabilities of the seeds were 54% higher for *R. officinalis*, 58% for *F. ericoides* and 54% for *F. thymifolia*. Higher seed survival should increase seedling establishment of the studied species.

VIII. Phylogenetic relationships of the genus *Fumana* based on the phylogenetic reconstruction of the two plastid markers confirmed the monophyly. Results do not support the traditionally established infrageneric divisions, but confirm the presence of two main groups of species.

IX. Divergence times of *Fumana* took place about 16.97 Ma ago (24-10 Ma), with a divergence of major clades between the Middle and Late Miocene (15.61-8.8 Ma).

X. Ancestral character states were analysed in 9 different morphological characters and significant ancestral states were found in five cases (leaf form and leaf margin, glandular trichomes, diaspore and seed mucilage secretion). This suggests an adaptation to the environment in the Mediterranean climate.

XI. Seed mucilage in the genus *Fumana* is most likely the ancestral character state, having devolved from a strong mucilage secretion to a reduced and almost absent form of mucilage in 4 of the 19 studied species.

XII. Seed mucilage in the family of the Cistaceae seems to be the most likely ancestral character state. Furthermore seed mucilage seems to have developed from pectin mucilage to cellulosic mucilage in the genus *Fumana* and to the total loss of seed mucilage in *Cistus*.

XIII. We found a pattern in the Cistaceae from species with strong mucilage secretion of cellulosic type, low RGR values, short seed longevities and big seed sizes that characterize the oldest genus (i.e. *Fumana*) towards the most modern species with no mucilage secretion, higher RGR and seeds with longer viability and smaller sizes in the genus *Cistus*.